# Relationship of Ammonia to Necrosis of Pepper Leaf Tissue During Colonization by Xanthomonas vesicatoria

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Journal Series Paper No. 4052, IFAS.
Accepted for publication 14 February 1972.

ABSTRACT

Ammonia accumulated in nutrient broth cultures during growth of *Xanthomonas vesicatoria*. Ammonia also accumulated in inoculated leaves of a pepper cultivar that was susceptible to a strain of *X. vesicatoria*, but did not in inoculated leaves of a pepper breeding line that was hypersensitive to the bacterium. Both types of pepper leaves were damaged by amounts of $NH_3$, generated from $NH_4Cl$, that were found in inoculated susceptible leaves after necrosis. Sensitivity of both types of pepper leaves to $NH_3$ from $NH_4Cl$ was similar, also. With inoculated susceptible leaves, a positive correlation was obtained between $NH_3$ accumulation and electrolyte leakage when plants received nitrogen fertilization. Necrosis occurred without significant increases in $NH_3$ in N-deficient leaves inoculated with the bacterium. Thus, ammonia accumulation was not necessary for necrosis of hypersensitive, or susceptible, leaf tissue following bacterial inoculation, and probably occurred after leakage of nitrogenous materials from the protoplasm.

Phytopathology 62:882-886.

*Additional key words:* ammonia toxicity, bacterial multiplication.

Leaf-spotting phytopathogenic bacteria have the potential to incite either a susceptible or hypersensitive reaction in leaf tissues of many plants (6). The hypersensitive reaction probably has the effect of restricting the host range of these pathogens. *Xanthomonas vesicatoria* (Doidge) Dows., the pathogen of bacterial spot of pepper (*Capsicum annuum* L.) incites either a susceptible or hypersensitive reaction in pepper, depending upon the cultivar and isolate. Hypersensitivity was associated with resistance under field conditions and is conditioned by a single gene (2).

Many events are probably involved in necrosis of leaf parenchyma in susceptible as well as hypersensitive reactions. Necrosis of susceptible parenchyma occurs at bacterial populations approximately 100 times greater than those in hypersensitive parenchyma (11). Either different

factors are involved in necrosis of the two types of leaf tissue, or a difference exists in the sensitivity to, or production of, a single factor leading to necrosis.

Volatile materials from nutrient agar cultures of several phytopathogenic bacteria caused necrosis of both susceptible and hypersensitive pepper leaf tissue (4). Ammonia is one of the constituents of the volatile products, and is toxic to plants (10). Therefore, in a search for factors involved in necrosis of plant tissue by pathogenic bacteria, an evaluation of $NH_3$ as a pathotoxin seemed necessary.

Lovrekovich et al. (7, 8) suggested that $NH_3$ was involved in necrosis of hypersensitive, as well as susceptible, combinations of tobacco with certain pseudomonads. Even though a correlation existed between $NH_3$ and necrosis, such correlations did not distinguish between cause and effect. In this paper, the role of $NH_3$ in necrosis of hypersensitive and susceptible pepper leaf tissue after inoculation with *X. vesicatoria* was studied. An abstract of part of this work was previously published (13).

MATERIALS AND METHODS.—*X. vesicatoria*, strain E-3, was selected for leaf inoculations and was cultured in nutrient broth for 24 hr, then centrifuged from the medium. The pellet was resuspended in sterile distilled $H_2O$ and made to a concentration of $10^8$ cells/ml. Inoculations were accomplished by injection-infiltration of the intercellular areas of leaves with a hypodermic syringe. Multiplication of bacteria in leaf tissue, or cultures, was determined at intervals by a dilution plate technique (11). Leaf extracts, used as a medium, were obtained as previously described (12).

Plants of the cultivar, Early Calwonder (susceptible to E-3), and the breeding line, 23-1-7 (hypersensitive to E-3), were grown in steam-sterilized soil in pots in a greenhouse. Fully expanded, but nonsenescent, leaves were used in all experiments. For most tests, plants were fertilized with a commercial 6:6:6 fertilizer as needed to maintain vigor. When plants were needed for high and low N experiments, plants were watered (500 ml/pot) twice weekly with a complete modified Knop's 4-salt solution (5). This was continued until first flower formation, after which half the plants were watered twice weekly with Knop's solution with N; and half, without N. The latter schedule was continued 4 weeks before using the plants in experiments.

Ammonia evolution from inoculated susceptible and hypersensitive leaves was determined from entire leaves enclosed in 250-ml disposable petri dishes while still attached to plants. Small slots were cut in the dishes to allow the petioles to remain attached. The dishes were held in place and closed by clamps attached to ring stands. Fully expanded, nonsenescent leaves, as uniform in size and maturity as possible, were selected. Air was drawn over the leaves by attaching plastic tubing from each plate to an aspirator. The airflow was estimated as ca. 1 liter/min. The air passing over the leaves bubbled through 25 ml of 2% $H_3BO_3$ which trapped $NH_3$. Controls included leaves infiltrated with $H_2O$ and dishes without leaves. These experiments were completed in a laboratory room with an average temperature of 27 ± 2 C.

In all experiments, injury of leaves was assessed from electrolyte leakage. This was associated with $NH_3$ accumulations in the leaf tissue. A half-leaf technique was used in that half of a leaf was used for electrolyte leakage and half for $NH_3$ determinations. Approximately 0.5 g fresh wt for each type of leaf was used in each sample for analysis. The proper weight was obtained by cutting 18 discs, 16 mm in diam, of 23-1-7 leaves and 15 discs of Early Calwonder leaves.

Electrolyte leakage values were obtained from electrical conductance of distilled $H_2O$ in which the leaf discs were suspended (3). Measurements were made with a RC 16B2 conductivity bridge and a cell with K = 0.01. Measurements obtained immediately after suspending the leaf discs were subtracted from measurements obtained after 4-hr soaking.

Ammonia in leaf samples was determined by modifications of the Barker & Volk method (1). Each leaf tissue sample (0.5 g fr wt) was frozen and triturated in 15 ml distilled $H_2O$. Five ml of borate buffer were added to an 100-ml tube containing the above suspension. The tube was immediately stoppered and placed in a 60-C waterbath. Air was bubbled through the mixture and into 25 ml of 2% $H_3BO_3$ solution for 30 min which trapped evolved $NH_3$. The amount of ammonia trapped in boric acid was determined with the use of Nessler's reagent. Color change was estimated with a Klett colorimeter.

To determine sensitivities of pepper leaf tissue to $NH_3$, excised leaves were exposed to $NH_3$ vapors in a 250-ml petri dish. Ammonia was generated by adding various amounts of $NH_4Cl$ to 20 ml of borate buffer at pH 10. The borate buffer was placed in a 90-mm plastic dish inside the large petri dish, and a 120-mm diam Whatman No. 2 filter paper was placed over the small dish. A detached leaf was then placed over the filter paper, and the large dish was sealed for 2 hr. Controls consisted of similar preparations except $NH_4Cl$ was excluded.

The ammonia in 1-ml samples of culture media was determined as previously described for leaf tissue. The samples consisted of supernatant after centrifugation to remove bacteria. Cultures were incubated at 30 C.

All experiments included three replicates and were repeated at least once with most experiments repeated 3 times.

RESULTS.—*Ammonia formed in vitro.*—Twenty-four isolates of *X. vesicatoria* were tested for production of $NH_3$ in nutrient broth cultures. These isolates differed in specificity of pathogenicity in leaves of the two pepper lines. All isolates produced $NH_3$. Ammonia increased in nutrient broth during growth of *X. vesicatoria*, strain E-3, during a 58-hr period (Table 1). Increases began just before concentration of the bacteria reached $10^8$ cells/ml. In three experiments of this type, $NH_3$ in the medium did not increase appreciably until the concentration of bacteria approached $10^8$ cells/ml.

Very little ammonia formed during bacterial

TABLE 1. Formation of $NH_3$ by *Xanthomonas vesicatoria* cultured in various media

| Hr | No. bacteria/ml | Ammonia (μg/ml) Nutrient broth + 1% glucose | Nutrient broth | Leaf extract |
|---|---|---|---|---|
| 0 | $10^3$ | 34.9 | 43.5 | 29.1 |
| 22 | $10^5$ | 23.9 | 20.8 | 23.4 |
| 27 | $10^6$ | 33.7 | 27.8 | 28.6 |
| 46 | $10^8$ | 28.6 | 55.0 | 34.7 |
| 58 | $10^9$ | 48.9 | 101.4 | 49.3 |

growth in extracts from pepper leaves, or in nutrient broth with 1% glucose. Multiplication of the bacterium was equally as rapid in both media as in nutrient broth alone. Thus, nutrient conditions during growth of *X. vesicatoria* influenced $NH_3$ production.

*Bacteria and susceptible leaf tissue.*—Injury of pepper leaf tissue after inoculation with bacteria was quantitated by measurement of the electrolyte leakage. After inoculation with suspensions of $10^8$ cells/ml of strain E-3, there was a progressive increase of the electrolyte leakage in susceptible tissue during a 96-hr period (Table 2). This was associated with an increase in bacterial population and pH of the inoculated leaf tissue.

Ammonia also increased in inoculated leaves over the 96-hr period. After 72 hr, leaves were completely collapsed. Most of the $NH_3$ that formed in the leaves remained there during the first 72-hr period. After 72 hr, most of the $NH_3$ was evolved and trapped outside of the leaf tissue, with very little increase in $NH_3$ in the leaves. Ammonia in the leaf tissue was positively correlated (r = .98) with electrolyte leakage. Electrolyte leakage, however, increased 37% (59 to 81 μmhos) during the first 24 hr without a corresponding increase in $NH_3$.

*Bacteria and hypersensitive tissue.*—After inoculation of hypersensitive tissue with a suspension of $10^8$ cells/ml of strain E-3, electrolyte leakage increased to a maximum during the following 12 hr (Table 3). During this period, the bacterial population in the leaf tissue approximately doubled. Ammonia in the hypersensitive tissue increased little during the 12 hr period after inoculation, and little was evolved. This was reflected by no change occurring in the pH values of the leaf tissue. Thus, no correlation (r = .02) existed between electrolyte leakage and $NH_3$ in hypersensitive leaf tissue.

*$NH_3$ toxicity to susceptible and hypersensitive leaf tissue.*—Susceptible and hypersensitive leaf tissues were treated with $NH_3$ generated from $NH_4Cl$. After treatment, electrolyte leakage was positively correlated with $NH_3$ accumulated in susceptible and hypersensitive leaf tissues (r = .91 and .85, respectively) (Table 4). A positive correlation was true whether one quantity of $NH_3$ vapors were used and the time of exposure varied, or several quantities of $NH_3$ vapors were used and the exposure time held constant. An increased pH value of the tissue was associated with higher $NH_3$ levels in the tissue.

Confidence intervals were calculated for lines of regression of the $NH_3$ in the tissue, from $NH_4Cl$, and the resulting electrolyte leakages. This was computed for both hypersensitive and susceptible tissues. The $NH_3$ sensitivity of hypersensitive and susceptible leaves seemed to be similar, since the line of regression of hypersensitive tissue fell within the confidence intervals calculated for the line of regression of susceptible tissue, and vice versa. Thus, differences in disease reaction cannot be due to differences in $NH_3$ sensitivity to the two types of tissue.

Pepper tissue was injured by accumulated levels that were also attained during colonization by strain E-3 in susceptible tissue. Therefore, injury of susceptible tissue after inoculations with *X. vesicatoria* seemed to be associated with $NH_3$ toxicity.

*Association of ammonia and injury in high and low nitrogen leaves.*—Experiments were undertaken with pepper plants, susceptible to strain E-3, grown under two levels of nitrogen fertilization. At the time of inoculation, the total N on a fresh weight basis in the high N (HN) leaves was 4.97%, and 3.08% in the low N (LN) leaves. Alcohol soluble N on a fresh weight basis was 0.16 and 0.09%, respectively.

HN leaves were injured more rapidly after

TABLE 2. Changes in susceptible pepper leaves during colonization by *Xanthomonas vesicatoria* (all figures are means of three determinations)

| Hr | No. bacteria | pH | | Electrolytes[a] | | $NH_3$ Evolved[b] | | $NH_3$ Tissue[a] | |
|---|---|---|---|---|---|---|---|---|---|
| | | Inoculated | Control[c] | Inoculated | Control | Inoculated | Control | Inoculated | Control |
| | × $10^6$ | | | μmhos | μmhos | μg | μg | μg | μg |
| 0 | 0.3 | 5.5 | 5.5 | 59.0 | 51.9 | | | 10.1 | 13.6 |
| 24 | 10.0 | 5.8 | 5.8 | 81.0 | 40.7 | 28.4 | 26.9 | 4.2 | 4.9 |
| 48 | 34.0 | 6.0 | 5.8 | 167.5 | 38.3 | 24.2 | 15.2 | 162.1 | 8.2 |
| 72 | 51.0 | 6.5 | 5.6 | 397.0 | 53.6 | 47.5 | 11.1 | 511.3 | 21.1 |
| 96 | 44.0 | 6.8 | 5.8 | 514.0 | 40.3 | 367.3 | 16.1 | 518.0 | 19.4 |

[a] Values from 0.5 g fresh wt leaf tissue.
[b] Values from an entire leaf, and represent $NH_3$ trapped during the preceding 24-hr period.
[c] Leaves infiltrated with water.

TABLE 3. Changes in hypersensitive pepper leaves during colonization by *Xanthomonas vesicatoria* (all figures are means of three determinations)

| Hr | No. bacteria | pH | | Electrolytes[a] | | $NH_3$ Evolved[b] | | $NH_3$ Tissue[a] | |
|---|---|---|---|---|---|---|---|---|---|
| | | Inoculated | Control[c] | Inoculated | Control | Inoculated | Control | Inoculated | Control |
| | $\times 10^6$ | | | μmhos | μmhos | μg | μg | μg | μg |
| 0 | 0.6 | 5.8 | 5.8 | 72.9 | 76.3 | | | 34.0 | 30.0 |
| 3 | | 5.8 | 5.8 | 72.3 | 66.2 | 9.6 | 10.6 | 22.9 | 27.5 |
| 6 | | 5.6 | 5.8 | 162.3 | 62.6 | 9.9 | 10.8 | 20.7 | 25.7 |
| 9 | 1.2 | 5.8 | 5.8 | 360.0 | 49.3 | 13.3 | 20.4 | 19.7 | 20.9 |
| 12 | | 5.8 | 5.8 | 613.3 | 83.6 | 52.9 | 36.2 | 28.0 | 22.6 |

[a] Values from 0.5 g fresh wt leaf tissue.
[b] Values from an entire leaf, and represent $NH_3$ trapped during the preceding 3-hr period.
[c] Leaves infiltrated with water.

TABLE 4. Effect of different levels of $NH_3$ on electrolyte leakage, $NH_3$ content, and pH of susceptible and hypersensitive pepper leaves

| $NH_4Cl$[a] | Susceptible tissue | | | Hypersensitive tissue | | |
|---|---|---|---|---|---|---|
| | Electrolytes | $NH_3$ | pH | Electrolytes | $NH_3$ | pH |
| mg | μmho | μg | | μmho | μg | |
| 0 | 44 | 15 | 5.60 | 94 | 24 | 5.43 |
| 2 | 102 | 178 | 5.65 | 113 | 393 | 5.95 |
| 4 | 119 | 253 | 6.05 | 147 | 325 | 6.15 |
| 6 | 255 | 360 | 6.15 | 204 | 380 | 6.35 |
| 8 | 272 | 290 | 6.15 | 310 | 384 | 6.30 |
| 10 | 350 | 410 | 6.35 | 303 | 585 | 6.80 |

[a] Leaves were exposed for 2 hr to above solutions containing these concentrations of $NH_4Cl$ in 20 ml of borate buffer at pH 10.

inoculation than LN leaves. This was reflected by electrolyte leakage data over a 120-hr period (Table 5). A reason for this may have been because bacterial populations increased more rapidly, and reached higher levels in HN than in LN leaves. The differences in numbers at any one sampling date were not considered to be significant, but relative numbers were higher at each sampling period.

Again, a positive correlation (r = .95) between electrolyte leakage and $NH_3$ in the tissue occurred in HN leaves. However, low correlation (r = .53) existed between electrolyte leakage and $NH_3$ in LN leaves. Even though total electrolyte leakage was delayed and was lower in LN leaves as compared with HN leaves, the LN leaves were completely collapsed after 120 hr. Thus, an increase in ammonia does not seem to be essential for necrosis of pepper leaf tissue in the susceptible reaction.

DISCUSSION.—Certain differences existed in the data presented here and the data of Lovrekovich et al. (7, 8, 9). The μg $NH_3$/g fresh wt in pepper were not as high as in their work, but this could reflect differences in plants or in nitrogen fertility programs. The pH values during bacterial colonization also were not so high in pepper leaves as those in tobacco leaves. Therefore, most of the $NH_3$ formed was trapped, and did not evolve from the pepper leaf until after complete collapse of the tissue. The evolved $NH_3$ was not associated with a decrease in tissue $NH_3$. Therefore, evolved $NH_3$ probably did not contribute to injury of pepper tissue.

The origin of $NH_3$ in the tissue is open to question. Possibly, $NH_3$ increased as the result of host-cell enzyme action in autolysis. However, $NH_3$ increases were not noted when pepper leaf tissue was frozen and thawed, which occurred in check treatments. Ammonia formed in certain media after inoculation with *X. vesicatoria*, which indicates that $NH_3$ is a product of metabolism of this organism. The association of $NH_3$ production in vivo and in vitro with only high bacterial numbers, and not necessarily with leaf necrosis, suggest that $NH_3$ forms in the leaf as the result of bacterial action at high concentrations of bacteria.

Only small amounts of $NH_3$ formed in cultures of bacteria growing in intercellular extracts from pepper leaves. This could be because of low levels of soluble nitrogenous compounds on the cell wall surfaces, or because of high levels of carbohydrates which may be selectively utilized by the bacterium. At any rate, this fact raises a question concerning whether $NH_3$ is formed within the intercellular spaces before injury

TABLE 5. Association of electrolyte leakage with $NH_3$ in susceptible pepper leaves with low (LN) and high (HN) nitrogen content[a]

| Hr | Bacterial no. | | pH | | $NH_3$ | | Electrolytes | |
|---|---|---|---|---|---|---|---|---|
| | HN | LN | HN | LN | HN | LN | HN | LN |
| | $\times 10^6$ | $\times 10^6$ | | | μg | μg | μmhos | μmhos |
| 0 | 2.6 | 3.0 | 5.8 | 5.8 | 46.6 | 36.9 | 83 | 43 |
| 24 | 29.0 | 24.0 | 5.9 | 5.8 | 39.1 | 36.9 | 80 | 50 |
| 48 | 100.0 | 84.0 | 5.9 | 5.7 | 60.3 | 30.4 | 135 | 88 |
| 72 | 46.0 | 34.0 | 6.1 | 5.9 | 237.7 | 33.0 | 247 | 80 |
| 96 | 120.0 | 75.0 | 6.1 | 5.8 | 510.3 | 38.9 | 543 | 131 |
| 120 | | 40.0 | | 5.8 | | 65.1 | | 396 |

[a] Each figure represents the mean of three determinations. The $NH_3$ and electrolytes were determined from 0.5 g fresh wt.

occurs, or after injury results in release of nitrogenous compounds from the cell.

A plausible explanation of the mechanism of specificity of pathogenesis must be accepted providing that $NH_3$ toxicity is the cause of necrosis in both hypersensitive and susceptible combinations, as suggested by Lovrekovich et al. (7, 8). They postulate that specificity was due to differences in the time necessary for accumulation of toxic amounts of $NH_3$ in hypersensitive and susceptible tissues. According to their data, $NH_3$ accumulates more rapidly in hypersensitive tissue than in susceptible tissue after inoculation. However, with equal numbers of bacteria in both types of tissue, it seems difficult to explain the appearance of $NH_3$ sooner in hypersensitive tissue than in susceptible tissue.

In this work, specificity of pathogenicity on the two plant types could not be explained on the basis of $NH_3$ accumulation in the leaves. Some factor other than $NH_3$ apparently operates to determine specificity. A major prerequisite for susceptibility seemed to be a characteristic that allows the pathogen to multiply to ca. $10^8$ cells/25 $mm^2$ of leaf tissue. Ammonia apparently formed after the pathogen multiplied to that high concentration in the leaves and after injury occurred. Thus, the position of Lovrekovich et al. (7, 8) is difficult to accept for the *X. vesicatoria*-pepper system.

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