# Calcium Suppression of Electrolyte Loss from Pepper Leaves Inoculated with Xanthomonas vesicatoria

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ABSTRACT

Suppression of electrolyte loss from inoculated leaves of *Capsicum annuum* cultivars hypersensitive and susceptible to race 2 (pepper strain) of *Xanthomonas vesicatoria* was obtained using inoculum ($10^8$ cells/ml) prepared with 0.2 N calcium nitrate. Lower concentrations of calcium nitrate in inocula were progressively less effective. No detrimental influence of any concentration of calcium nitrate used on viability of bacteria in vivo was detected. Comparable results were obtained when calcium nitrate in corresponding concentrations was injected into hypersensitive pepper leaves 16 hr prior to inoculation with bacteria. The chloride forms of calcium and strontium, but not magnesium, were as effective as calcium nitrate. Pretreatment of test leaves with $10^{-3}$ M or lower concentrations of uranyl acetate did not suppress electrolyte loss as effectively as calcium. Calcium was generally ineffective in suppressing electrolyte loss from hypersensitive and susceptible leaf tissue treated with ammonia or volatile substances from bacterial cultures. Phytopathology 61:484-487.

Race 2 (pepper strain) of *Xanthomonas vesicatoria* (Doidge) Dows. (3) at inoculum concn of $10^7$ cells/ml, or greater, induced distinguishable responses in selected cultivars of pepper (*Capsicum annuum* L.) (14). An abrupt increase in electrolyte loss is elicited within 12 hr after inoculation from leaves of resistant plants and prior to the appearance of visible symptoms (1). In susceptible leaves, this same phenomenon occurs, but more gradually and only after an extended incubation period. Early electrolyte loss followed by rapid tissue collapse in leaves of resistant plants is considered a hypersensitive response, but is not accompanied by detectable increase in bacterial population in vivo. The more gradual increase of electrolyte loss from susceptible leaves follows the same general pattern as bacterial multiplication but precedes it by 6-12 hr. The causative nature of hypersensitivity, dependent on a minimal concn of bacteria in vivo but not on bacterial multiplication, may be intimately related to inherent resistance of plants to bacterial infection (7).

Volatile products evolved from in vitro cultures of *X. vesicatoria* (XV) (2) and ammonia (A. A. Cook, *unpublished data*) induce electrolyte loss from healthy leaves of hypersensitive and susceptible peppers. Some cations, notably calcium (4), and uranyl acetate (6, 11) influenced electrolyte loss from oat tissues susceptible to the pathotoxin victorin (16). The following study was conducted to elucidate the mechanism(s) involved in the hypersensitive response to bacterial infection.

MATERIALS AND METHODS.—Mature leaves from plants of a breeding line of *C. annuum* homozygous for hypersensitivity to race 2 of the pepper strain of XV and designated 23-1-7 (HYP) were used throughout this study. Comparable leaves from susceptible Early Calwonder (SUS) plants also were used. Plants were placed in a growth room maintained at approx 30 C prior to inoculation, and allowed to remain there until test leaves were harvested. Inoculated plants were exposed to continuous fluorescent light of approx 600 ft-c during incubation.

Bacterial cells for inoculum were harvested by centrifuging 24-hr nutrient broth shake cultures 10 min at 1,475 *g*. Pelleted cells were resuspended in sterile, distilled water. Inocula were prepared immediately before use by addition of aliquots of a standard, concd suspension of cells in distilled water to the test medium. Final concn of bacterial cells in test suspensions was approximated by addition of an aliquot comparable to that which gave 50% transmittance at 650 mμ in the same quantity of distilled water. Where indicated, test solutions of various concentrations were injected into healthy leaves at selected time intervals prior to injection of bacterial cells suspended in distilled water.

Exposure of leaves to volatile materials from bacteria in vitro and ammonia was accomplished in the laboratory at ambient light and a temperature of approx 25 C. Uncovered petri dish cultures (9 cm) of XV on nutrient agar, supplemented with 1% casein hydrolyzate, were placed inside 14-cm plastic petri dishes. Test leaves, excised immediately before treatment, were placed on sheets of filter paper laid across the opened, smaller petri dishes containing the bacteria, and the lid of the larger petri dish was replaced. The spacing nibs were removed from the underneath side of the lids of the larger petri dishes, which made them essentially airtight. For exposure to ammonia, 20 ml of buffer (pH 10) (10) was placed in the bottom half of the smaller petri dish, to which was added an aliquot (2 ml or less) of an appropriate dilution of $NH_4Cl$ suspended in water. After the $NH_4Cl$ was added, the lid of the larger petri dish was replaced immediately and a weight placed on it until termination of the exposure.

Electrolyte losses were assessed by cutting 18 discs 16 mm in diam from test HYP leaves, and 15 discs of the same diam from SUS leaves (approx 0.5 g wet wt of healthy leaf tissue in both cases). Discs were submersed in 20 ml glass distilled water, and the conductivity in micromhos of the suspending water was immediately established with an RC 16B2 conductivity bridge and cell with $K = 0.01$ (Beckman Instruments). The conductivity of the suspending water was again determined after 4 hr without agitation, and the increase in conductivity used for evaluating electrolyte loss.

RESULTS.—Test leaves from HYP plants inoculated

with XV ($10^8$ cells/ml) suspended in various concentrations of calcium nitrate and other inorganic chemicals were harvested 6-8 hr after inoculation. Termination of the incubation period was determined by first appearance of turgidity loss in control leaves inoculated at the same time with a similar concentration of XV suspended in water. Compared with electrolyte loss from leaves inoculated with bacteria suspended in water only, electrolyte loss was progressively suppressed by increasing concn of calcium nitrate (up to 0.2 N) in the inoculum (Fig. 1). Electrolyte loss from inoculated leaves of SUS plants also was progressively suppressed with increase in concn of $Ca(NO_3)_2$ in the inoculum (Fig. 2). A concn of 0.05 N $Ca(NO_3)_2$ in the inoculum had little effect on electrolyte loss, and even 0.2 N caused less suppression of electrolyte loss from SUS leaf tissue than from HYP. The apparent increase in suppression with increase in incubation time in SUS leaves actually resulted from comparatively greater electrolyte loss from control leaves inoculated with bacteria suspended in water only. There was no indication that calcium concentration in the inocula influenced bacterial multiplication in vivo.

Suppression of electrolyte loss from HYP leaves also was achieved when $Ca(NO_3)_2$ solutions were injected into leaves 16 hr prior to introduction of bacteria ($10^8$ cells/ml) suspended in water (Fig. 3). A similar pattern of electrolyte loss suppression was obtained when $CaCl_2$ or $SrCl_2$ was injected before the challenge inoculation with live bacteria, but $MgCl_2$ had no effect. Uranyl acetate concn of $10^{-3}$ M or less were less effective than calcium. Treatment prior to inoculation did not notably affect numbers of viable bacteria recovered from test leaves (Table 1).

Test leaves of HYP and SUS injected with three concn of $Ca(NO_3)_2$ were excised after signs of water-soaking had disappeared, and exposed immediately to volatiles that emanated from in vitro petri cultures of XV. Leaves were exposed for 8 hr to cultures incubated 34 hr before use, but only for 4 hr to cultures previously incubated 40 hr before electrolyte loss was evaluated. All solutions of calcium partially suppressed electrolyte loss from HYP leaves exposed to 34 hr cultures of XV (Fig. 4) but had no effect, or enhanced, electrolyte loss from leaves exposed to 40-hr cultures. Prior injection of calcium caused greater electrolyte loss from SUS leaves exposed to bacterial cultures of both ages than previous treatment with water only.

Leaves of both plant types, excised after water-soaking had subsided following injection with three concn of $Ca(NO_3)_2$, were exposed for 8 hr to each of three concn of ammonia. Prior injection of calcium generally was ineffective in altering electrolyte loss compared to electrolyte loss from leaves injected with water only (Table 2). While 0.2 N calcium concn did influence electrolyte loss from HYP leaves at each ammonia concn, this effect was in no way comparable to that combined following inoculation with live bacteria.

DISCUSSION.—Inoculation of hypersensitive pepper leaves with selected pathogenic cultures of XV has been found to induce electrolyte loss prior to the appearance of visible symptoms. The nature of the prerequisite cellular condition(s) has not been definitely established, but alteration of cell membrane permeability is a common characteristic of diseased plant cells (17). Doupnik (4) and Hanchey (6) have reported that calcium and uranyl acetate suppress electrolyte loss from oat tissues normally susceptible to the pathotoxin victorin. In neither case was there clearly distinguished an increased resistance of host tissue to induced electrolyte loss, but it was suggested earlier (16) that permeability changes may be a causative factor for increased respiratory activity. An effect on host tissue was demonstrated in the study herein reported, and was induced by pretreatment with calcium 16 hr before inoculation. In preliminary experiments, suppression of electrolyte loss was noted by injection of calcium nitrate (0.2 N) 24 and 48 hr before inoculation with bacteria.

Electrolyte loss was not suppressed from either HYP or SUS leaves pretreated with calcium and exposed to the 2 lowest concn of ammonia used. Analyses of hypersentitive leaves treated with these concentrations of ammonia gave yields of $NH_3$ comparable to those obtained from similar leaves infiltrated with $10^8$ cells/ml

TABLE 1. Numbers of colonies of *Xanthomonas vesicatoria* ($\times 10^5$) recovered[a] from hypersensitive pepper leaves 8 hr after infiltration with bacteria suspended in three concentrations of four chemicals and in distilled water

| | Chemical concentration | | | |
|---|---|---|---|---|
| | 0.05 N | 0.1 N | 0.2 N | $H_2O$ |
| $Ca(NO_3)_2$[b] | 3.7[c] | 2.7 | 5.1 | 5.7 |
| $CaCl_2$ | 2.1 | 2.6 | 2.5 | 3.3 |
| $SrCl_2$ | 1.0 | 0.7 | 1.1 | 1.3 |
| $MgCl_2$ | 3.1 | 2.2 | 1.7 | 2.3 |
| $UO_2(C_2H_3O_2)_2$[d] | 2.1 | 5.7 | 0.8 | 1.9 |

[a] Serial dilutions made from suspension of 48 $mm^2$ leaf tissue triturated in 2 ml sterile saline (0.85%).
[b] Separate inoculum batch prepared for dilution series of each chemical and related water inoculations.
[c] Average of three replicates.
[d] Uranyl acetate concentration $10^{-5}$, $10^{-4}$, $10^{-3}$ M, respectively.

TABLE 2. Electrolyte loss[a] from healthy pepper leaves exposed for 8 hr to three concentrations of ammonia[b] after infiltration with three concentrations of calcium nitrate.

| Quantity $NH_4Cl$ | $Ca(NO_3)_2$ concentration | | |
|---|---|---|---|
| | 0.05 N | 0.1 N | 0.2 N |
| g | *Hypersensitive leaves* | | |
| 0.002 | 90[c] | 108 | 142 |
| 0.004 | 108 | 103 | 127 |
| 0.008 | 87 | 87 | 66 |
| | *Susceptible leaves* | | |
| 0.002 | 115 | 101 | 101 |
| 0.004 | 92 | 70 | 91 |
| 0.008 | 112 | 102 | 96 |

[a] Presented as per cent of electrolyte loss, measured in micromhos, from control leaves infiltrated with distilled water prior to exposure.
[b] Ammonia liberated by adding indicated quantity of $NH_4Cl$/100 $cm^3$ to 20 ml buffer (pH 10).
[c] Average of nine replicates.

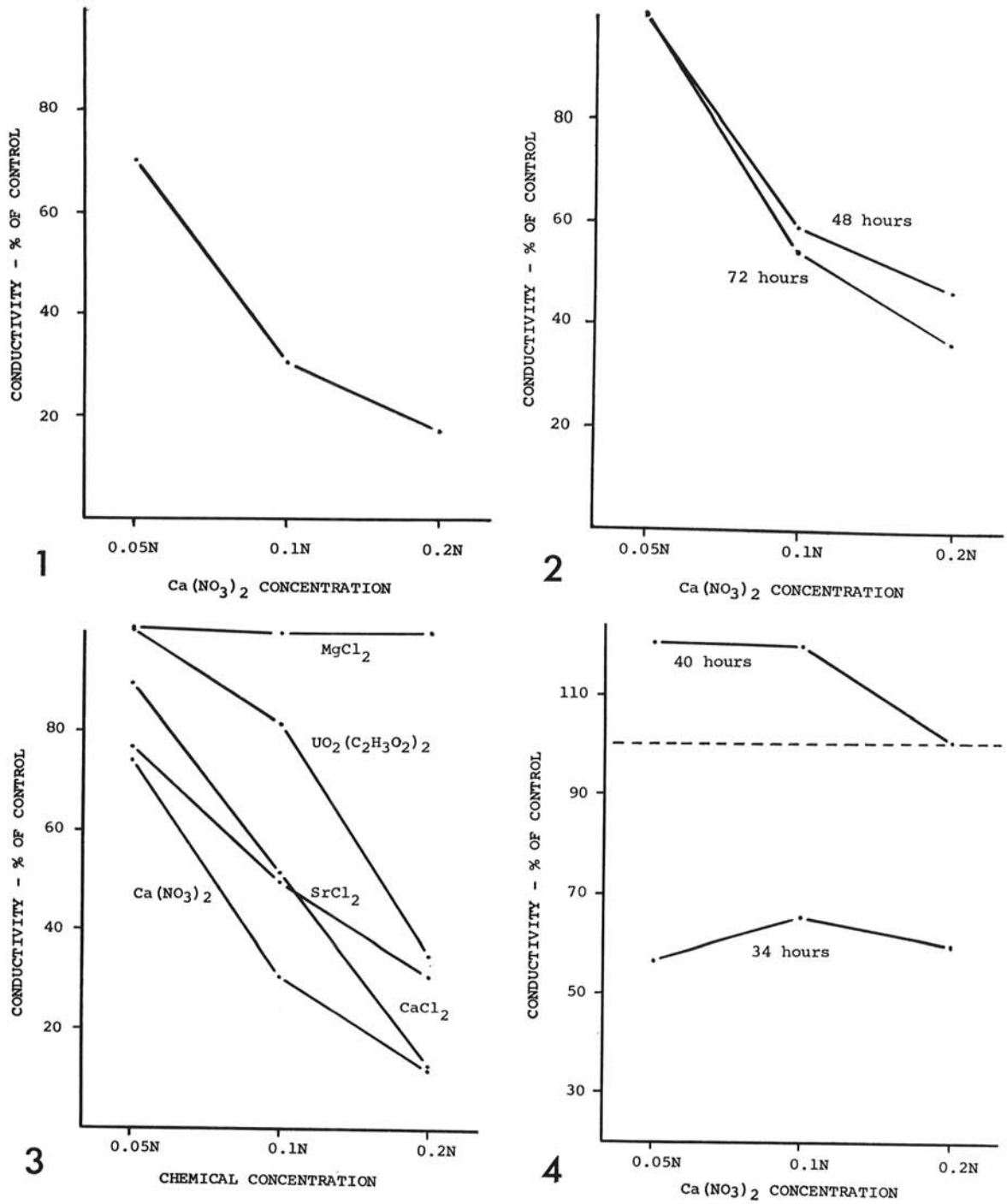


**Fig. 1-4.** **1)** Suppression of electrolyte loss from hypersensitive pepper leaves 8 hr after inoculation with *Xanthomonas vesicatoria* ($10^8$ cells/ml) by three concentrations of calcium nitrate in the inoculum expressed as per cent of water-inoculum control. Average of 12 replicates. **2)** Suppression of electrolyte loss from susceptible pepper leaves 48 and 72 hr after inoculation with *X. vesicatoria* ($10^8$ cells/ml) by three concentrations of calcium nitrate in the inoculum expressed as per cent of water-inoculum control. Average of three replicates. **3)** Suppression of electrolyte loss from hypersensitive pepper leaves 8 hr after inoculation with *X. vesicatoria* ($10^8$ cells/ml) by five chemicals infiltrated into leaves 16 hr prior to inoculation and expressed as per cent of appropriate water-inoculum control. Uranyl acetate concentrations were $10^{-5}$, $10^{-4}$, and $10^{-3}$ M, respectively. Average of three replicates. **4)** Electrolyte loss from hypersensitive pepper leaves, infiltrated with three concentrations of calicum nitrate, after 8-hr exposure to volatile material (s) from in vitro cultures of *X. vesicatoria* incubated 34 and 40 hr before use.

bacteria (15). With the highest concentration of ammonia, which caused excessive $NH_3$ accumulation in leaves compared to bacterial inoculation, loss of electrolytes was reduced by calcium and related to concentrations used in pretreatment. Suppression was less marked than with live bacteria, as were differences between calcium concentration.

Even though it has been demonstrated that calcium, either as nitrate or chloride, will suppress electrolyte loss from leaf tissues inoculated with XV, it has not been established that such suppression is a general phenomenon effective against all phytopathogenic bacteria. This protective effect in HYP leaves may endure for at least 16 hr, and possibly more than 48 hr, prior to inoculation, and during 8-hr incubation or up to when leaf tissues exhibit first visible symptoms. The manner in which electrolyte loss suppression was effected by calcium was not determined, but presumably deterioration of cell membrane integrity was retarded (12). The effectiveness of calcium and ineffectiveness of magnesium in maintaining selective absorptive processes of some elements, viz., potassium, in plant cells have been reported (5). Victorin induces excessive loss of potassium from susceptible oat tissues, but the chemical elements involved in the hypersensitive response to bacterial infection remain unidentified. Suppression of tissue-macerating enzyme activity by magnesium and calcium, and particularly the associated nitrate anions (13), is presumptive evidence that hypersensitivity to bacterial infection involves processes unlike those associated with soft-rot diseases. That calcium did not afford comparable suppression of electrolyte loss from leaf tissue exposed to either volatiles from bacteria in vitro or ammonia is circumstantial evidence that neither of these materials is directly involved in cell membrane destruction associated with hypersensitivity to XV (8). The influence of light on the suppression of the hypersensitive response (9) assayed by electrolyte loss was not a major consideration in this study, but was found in preliminary experiments with calcium to be inconsequential.

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