Internal and external infections of fruits and seeds of peppers by *Xanthomonas campestris* pv. *vesicatoria*

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Fruit infection by *Xanthomonas campestris* pv. *vesicatoria* (Doidge 1920) Dye 1978 was monitored by scanning electron microscopy and by bacterial counts. Possible sites for bacterial penetration were through dead flowers and by proliferation of bacteria in the wart (small protuberance) area. Bacterial multiplication was observed in all warts of young, mature, and ripened fruits. Bacterial cells were bound to the fruit surface by fibrillar material. On the fruit surface, bacteria multiplied in small aggregates, submerged in slime. The slime consisted mainly of sucrose units. At later stages of disease development, the slime covered the entire fruit surface in young fruits. Typical scab symptoms appeared only in leaves of inoculated plants, whereas buds, flowers, and fruits of various sizes were symptomless, but later shed; shedding was strongest in young buds. In more mature and ripened fruits, bacterial numbers decreased and there was less shedding. *Xanthomonas campestris* pv. *vesicatoria* could be detected, in enrichment culture, in low numbers at the seed site (ovary) in inoculated fruits that did not shed.

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**Introduction**

*Xanthomonas campestris* pv. *vesicatoria* (Doidge 1920) Dye 1978 attacks pepper leaves and causes visible symptoms and shedding (7, 9, 10, 11). The pathogen enters via the leaf veins and, to a small extent, the stomata (18). Under the relatively hot summer conditions in Israel, visible fruit infections are rare (3) in contrast to conditions in other countries (11). Naturally infested pepper seeds serve as a source of inoculum for the establishment of the disease under appropriate environmental conditions (1, 4, 6, 8, 15). However, it is not known how seeds become contaminated.

The purposes of this study were to follow the infection of pepper fruits after inoculation with *X. campestris* pv. *vesicatoria* in the hope of determining possible fruit sites of entry and sources of seed contamination and to determine the infection process.

**Materials and methods**

**Plant inoculation**

*Xanthomonas campestris* pv. *vesicatoria* (R-3) and pepper plants (*Capsicum annuum* cv. 'Ma'or') (susceptible to bacterial scab (2)) were grown as previously described (9). Mature plants at the flowering stage were inoculated.

Inoculation was carried out by one of the following methods. (i) Mature plants at the flowering stage were kept in a humid chamber for 24 h, sprayed to runoff with a bacterial suspension containing 10⁷ colony-forming units (CFU) per millilitre, and incubated in this chamber at approximately 100% relative humidity and 32 ± 2°C for up to 10 days (5, 9). Buds, open flowers, very young fruits (less than 0.5 cm in diameter), and small fruits (greater than 0.5 cm in diameter) were each marked at the time of inoculations. (ii) Detached, mature green fruits were washed under a gentle stream of tap water for 10 min, inoculated by placing 0.1 mL of the bacterial suspension on the wart area, and kept with the wart side up for 8 days at 28 ± 2°C and at a 12 h illumination period at 100 W/m², in solid plastic humid chambers (40 × 30 × 20 cm) that were sealed with polyethylene. Control plants were sprayed with sterile tap water.

**Determination of bacterial populations**

Triplicate samples of three detached buds or flowers or three samples of 2-cm² area from young, green mature, or red mature fruits cut with a cork borer were kept in an ice bath and homogenized in 4.5 mL sterile 0.06 M potassium phosphate buffer, pH 7.0, using a fine-glass homogenizer (Kontes, U.S.A.). The homogenates were decimally diluted and 0.1-mL aliquots were spread with a glass rod on the surface of nutrient agar (Difco) supplemented with 10 g sucrose, 1.5 g CaCl₂, and 200 mg sodium deoxycholate per litre (the latter added after autoclaving) (NSD) (1). The inoculated plates were incubated at 30 ± 2°C for 72 h. Typical *X. campestris* pv. *vesicatoria* colonies were tested for pathogenicity (1).

Ten days after inoculation all previously marked fruits of different
sizes were sampled, washed for 30 min under a stream of tap water, immersed in 90% alcohol, and flamed in a laminar-flow hood. Fruits were opened aseptically and tissue samples (about 0.5 g) were taken from seed sites (ovary), beneath the wart and from the middle distance between these sites. The tissue samples were incubated in nutrient broth medium supplemented with 200 mg/L sodium deoxycholate (NBD) for 30 ± 2°C for 24 h. Aliquots of 0.1 mL were spread on NSD agar and incubated for an additional 72 h, and then the colonies were counted. Typical X. campestris pv. vesicatoria colonies were tested for pathogenicity (1).

Alternatively, all seeds from each fruit were aseptically harvested and transferred into 20 mL sterile 0.06 M potassium phosphate buffer,
Fig. 2. *X. campestris pv. vesicatoria* on the surface of young pepper fruit. (a) Bacteria embedded in a slime matrix 24 h after inoculation. ×6000. (b) At 48 h after inoculation. ×3000. (c) At 72 h after inoculation. ×50. (d) Insert of Fig. 2c showing bacteria embedded in the slime matrix. ×1700.

pH 7.0, in Erlenmeyer flasks and vigorously shaken for 3 h. The presence of *X. campestris pv. vesicatoria* in the supernatant was determined as above, directly on NSD agar.

**Paper chromatography**

Five days after inoculation, young and mature pepper fruits were washed with a fine brush in 2 L of double-distilled water. The solution was lyophilized to dryness and the pellet was hydrolyzed (1 M HCl, 100°C, 5 h) and then neutralized by 1 M NaOH. Paper chromatography was done on Whatman No. 42 filter paper (40 × 80 cm), using ammonia – water – ethyl acetate – propanol (5:1:3:1) or water–acetone–butanol (1:5:4) as solvents. After overnight running, a freshly prepared developing mixture of 0.5 mL anisaldehyde, 9 mL absolute ethanol, 0.5 mL concentrated sulphuric acid, and 0.1 mL glacial acetic acid was sprayed on. The chromatogram was then heated at 100°C for 10 min. Alternatively, a developer of 0.5 g benzidine dissolved in 20 mL glacial acetic acid and 80 mL absolute ethanol was used and the chromatogram was heated at 100°C for 15 min and then sprayed with 0.01 M HCl. Glucose, mannose, lactose, sucrose, fructose, and galactose were used as markers (20, 22).

**pH determination**

Fruit segments (five per fruit from 10 fruits) or 10 leaves were homogenized in an Omni-mixer (Sorval) and filtered through cheesecloth. The pH of the supernatant was determined with a pH meter.

**Scanning electron microscopy (SEM)**

Pepper buds, flowers, and fruits of the three types were examined by SEM at 24-h intervals during the first 6 days after inoculation. Ten samples from each type of plant part were prepared daily as described previously (18) and scanned with a Philips SEM 505 scanning electron microscope at 30 kV.

**Experimental design and statistical analysis**

All experiments were repeated two or three times with three to six replicates in each. The replicates, arranged in a randomized design, consisted of 10 plants, 5–10 plant parts, or 5 SEM stubs. The results given are from a representative experiment. Significance is indicated by $P \leq 0.05$ or by standard error.

**Results**

**Scanning electron microscopy of pepper fruit infection**

Young, mature, and ripened pepper fruits were inoculated with *X. campestris pv. vesicatoria*. Disease development was monitored during the 6 days following inoculation. Before inoculation, very few or no microorganisms were observed on fruit surfaces. Immediately after artificial inoculation, randomly dispersed bacterial cells were observed on the tissue surfaces. At 24 h after inoculation, observations were made at two sites, the wart area (Fig. 1a) and the fruit surface, since the mode of infection differs at these two locations. Dead dry flowers (Fig. 1b) provided many penetration sites in the form of holes and “caves.” Higher magnification showed many bacterial cells in these spaces (Fig. 1c). At 48 h after inoculation, many bacterial aggregates were seen in the wart area (Fig. 1d). Their number increased after 72 h (Fig. 1e); after 100 h most of the wart area was covered with bacteria. The bacterial mass appeared as if multilayered (Fig. 1f) with bacterial cells connected by a tridimensional fibrillar net (Fig. 1g). At 120 h after inoculation, the entire area was covered with bacteria embedded in a slime matrix (Figs. 1h, 1i).
Fig. 3. *X. campestris* pv. *vesicatoria* on the surface of mature pepper fruit. (a) Aggregates of bacteria on the fruit surface. ×330. (b) The same on the fruit base. ×180. (c) Insert of Fig. 3b showing bacteria concentrated in a defined zone. ×4300. (d) Insert of Fig. 3a showing bacteria near the defined zone. ×3000.

Similar SEM observations were made on the warts of very young fruits and those on mature or ripened fruits.

Different patterns of bacterial multiplication were observed on the surfaces of young and mature fruits. Many evenly distributed sites of bacterial multiplication were observed on the surface of young fruits, 24 h after inoculation. Multiplication was not massive, and the bacteria were embedded in a slime matrix (Fig. 2a). After 48 h, the bacteria—slime material had proliferated (Fig. 2b), and after 72 h the whole fruit was nearly covered with slime (Fig. 2c) embedding the bacteria (Fig. 2d). However, in no instance did the amount of bacteria visible on the surface (more than 100 sites were observed) ever reach the bacterial mass observed in the wart area.

On mature and ripened fruits, bacterial cells did not multiply on the entire fruit but were limited to round defined zones (Figs. 3a and 3b). It was only at these sites that bacterial multiplication inside the slime occurred within 24 to 48 h after inoculation. These areas became bigger with time, but their number did not increase. The number of bacteria embedded in the slime was small (Figs. 3c and 3d). Several attempts to detect *X. campestris* pv. *vesicatoria* cells at ovary sites within the fruit with SEM were unsuccessful.

**Multiplication of X. campestris pv. vesicatoria on pepper fruits**

Determination of pathogen population levels on the surface of young and mature fruits revealed no difference in bacterial numbers per unit area. Pathogen cell counts in the fruits increased from 10^3 CFU/cm^2 of tissue at the time of inoculation to approximately 10^5 CFU/cm^2 of tissue 4 days later. A different multiplication pattern was found in the wart, where bacterial cells increased during incubation and reached 10^6 CFU/cm^2 tissue after 4 days (Fig. 4).

**Paper chromatography of the slime on the inoculated fruit surface**

Figure 5 shows that the slime observed by SEM which covered the surface of both young and mature infected fruits was composed mainly of sucrose. This was verified by using different solvents and developers. Slime characteristics remained unchanged during the 8 days of the experiment.

**Changes in pH values in leaves and fruits after inoculation with X. campestris pv. vesicatoria**

Different patterns of pH change were detected in infected pepper leaves and fruits. In infected leaves, pH increased gradually with time until it differed from that of healthy leaves by nearly 1 pH unit. In young, mature, and ripened fruits, a decrease in pH was observed 48 h after inoculation. The pH later increased until it reached the level originally observed in young fruits (pH 6.3). In mature and ripened pepper fruits, the pH was lower 96 h after inoculation than that of healthy controls (Fig. 6).
Hours after inoculation

**FIG. 4.** Counts of *X. campestris* pv. *vesicatoria* from pepper fruits. Wart, ○; fruit surface of young (∆) and mature (▲) fruits.

**Fig. 5.** Drawing of paper chromatography of slime obtained from inoculated fruits.

**Effects of* X. campestris* pv. *vesicatoria* inoculation on vegetative and reproductive parts of the pepper plant**

When whole mature pepper plants were inoculated and examined after incubation, only the leaves displayed typical scab symptoms. Buds, flowers, and fruits at various stages of development showed no apparent symptoms of infection. However, shedding of these plant parts occurred; it was most severe at flower bud initiation and decreased in severity as the fruit ripened, paralleling the decrease in bacterial numbers. The number of bacteria detected internally in the fruits which remained on the plant was low (less than 10^3 CFU/fruit in severe cases) (Table 1).

To overcome this difficulty, a different type of experiment was performed on 25 detached pepper fruits. The pathogen was detected after liquid enrichment. Figure 7 shows internal infections of small, mature and full-size fruits; 30–60% of fruits were internally infected below the wart. Bacterial incidence inside the fruit decreased with the increase in distance from the fruit wart. Nevertheless, some seed sites (ovary) of each fruit type contained *X. campestris* pv. *vesicatoria* cells.

**Discussion**

Typical bacterial scab symptoms have rarely been observed on pepper in Israel (3). Infected seed is one of the known sources of *X. campestris* pv. *vesicatoria*, and there is suggestion that the seeds become infested from diseased fruit (4, 6). The basic question of whether this infestation originates from penetration of the pathogen into the intact fruits or during processing by the seed industry remains unanswered (19).

By artificial inoculation of pepper plants and detached fruits, it was shown that the pathogen has the ability to penetrate into the interior of developing fruit and seeds under favorable environmental conditions for disease development. The primary site of penetration was through the dead flower and, later, the main bacterial multiplication site was the wart, in which *X. campestris* pv. *vesicatoria* numbers were very high (more than 10^6 CFU/cm^2 fruit area). On the wart, bacteria were found in aggregates bound by a fibrillar matrix as observed in
known. In other areas of the fruit, bacterial multiplication was
The role of this matrix in disease development is not yet
SEM preparations; this matrix may prevent removal of bacteria
from other foliar bacterial pathogens as well (12, 14,
From the site. This was not true for populations on leaves (18).
limited. Penetration through flowers and fruits is
Typical scab symptoms on leaves only. The reproductive plant
parts examined, flowers and fruits of various sizes and buds,
displayed no symptoms. However, these plant parts tended to
be shed. Buds were the most susceptible to shedding. A
gradual decrease in shedding was observed with ripening of the
reproductive parts of the plant. At the same time, the patho-
genic bacterial population in these plant parts decreased. Shed-
ing of diseased leaves was previously attributed to an
accumulation of ethylene as a result of X. campestris pv. vesi-
catoria proliferation and disease progress (7, 21). The
more limited attack on fruits observed in Israel might be due in part
to a decrease in fruit pH after inoculation to a level that pre-
vents the pathogen from multiplying. A similar phenomenon
was observed in tomato plants infected with Pseudomonas
syringae pv. tomato, where the low pH of the fruits prevented
spect development (23).
Thus, it may be suggested that the visible phenomena occur-
ing during bacterial scab infection of pepper fruits are differ-
ent from those occurring on pepper leaves and there is a
possibility of seed infestation during disease development.

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monas campestris pv. vesicatoria in pepper seeds and roots in
symptomless and dry leaves in non-host plants and in the soil.

Table 1. Effects of X. campestris pv. vesicatoria inoculation* of mature pepper plants* on response of various parts of
table part

<table>
<thead>
<tr>
<th>Plant part</th>
<th>Typical bacterial scab symptoms</th>
<th>Shedding (%)</th>
<th>No. of X. campestris pv. vesicatoria on plant surface (cm²)</th>
<th>Development of diseased plants (%)</th>
<th>CFU of X. campestris pv. vesicatoria in seeds of infected fruits*</th>
<th>Pathogenicity tests of isolates obtained from seeds*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>2.86 ± 0.03 ±</td>
<td>17 ± 4</td>
<td>4.73 ± 1.28 × 10⁶</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Buds</td>
<td>0</td>
<td>86 ± 7</td>
<td>3.61 ± 0.48 × 10⁴</td>
<td>92 ± 2</td>
<td>4.03 ± 0.19 × 10³</td>
<td>+</td>
</tr>
<tr>
<td>Flowers</td>
<td>0</td>
<td>46 ± 10</td>
<td>2.44 ± 0.17 × 10⁴</td>
<td>87 ± 4</td>
<td>8.84 ± 0.44 × 10⁴</td>
<td>+</td>
</tr>
<tr>
<td>Small fruits*</td>
<td>0</td>
<td>28 ± 8</td>
<td>1.86 ± 0.17 × 10⁴</td>
<td>7 ± 2</td>
<td>4.6 ± 0.2 × 10⁴</td>
<td>+</td>
</tr>
<tr>
<td>Small fruits*</td>
<td>0</td>
<td>4 ± 1</td>
<td>8.44 ± 0.98 × 10⁴</td>
<td>6 ± 1</td>
<td>6.8 ± 0.4 × 10⁴</td>
<td>+</td>
</tr>
<tr>
<td>Large fruits*</td>
<td>0</td>
<td>0</td>
<td>4.81 ± 0.79 × 10⁴</td>
<td>6 ± 3</td>
<td>3.1 ± 0.9 × 10⁴</td>
<td>+</td>
</tr>
</tbody>
</table>

*Whole plants were inoculated with 10⁶ CFU/mL. The response of each plant part was measured separately.
*Plants with six true leaves. Twelve plants per replicate, five replicates.
The experiment was repeated three times. Data presented are from a representative experiment. ND, not determined.
*Seeds were obtained when each reproductive plant part of the 60 plants reached harvesting time.
*By inoculating two young pepper plants (four true leaves) per isolate with 1 × 10⁶ CFU/mL.
Index: 0, no symptoms; 3, more than 10 scab lesions per leaf (9).
Fruits larger than 8 and smaller than 30 mm.
*Fruits 2-8 mm in diameter.
Fruits larger than 30 mm.
*Full-size fruits (over 100 mm).

Fig. 7. Seed infestation inside pepper fruits after inoculation at the wart. ▲, small fruits; ○, medium fruits; ●, full-size fruits. Arrows indicate seed site (ovary) in each fruit type.


