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Survival of *Xanthomonas campestris* pv. *vesicatoria* in pepper seeds and roots in symptomless and dry leaves in non-host plants and in the soil

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Summary A population of *Xanthomonas campestris* pv. *vesicatoria* developed as endophytes in the leaves and rhizosphere of apparently symptomless plants grown under mist but not under dry conditions. The pathogen survived for long periods on, and could be isolated from, the surface of infested dried seeds, inoculated sandy loam soil, dried leaves, and the rhizosphere of pepper and of other non-host plants. In addition, small numbers of the pathogen survived for 18 months in a field previously cropped with pepper diseased with bacterial scab. Healthy nursery or mature plants developed symptoms while growing in soil containing infested leaves, either buried or placed on the soil surface.

Introduction

Xanthomonas campestris pv. *vesicatoria*, the causal agent of bacterial scab of tomato and pepper, is one of the most intensively investigated phytopathogenic bacteria. Most studies performed during the last 60 years have dealt with interactions of the bacterium with tomato plants and only a few with its relationship with pepper tissues[5,10,12,13,14,16,18,23]. The common knowledge on epidemiology and survival of the pathogen is controversial because *X. campestris* pv. *vesicatoria* is well known as a heterogeneous species. Strains which were isolated in different geographical areas differ in their physiological and pathogenic characteristics[8,9]. It was suggested by Klement[11] that isolates of *X. campestris* pv. *vesicatoria* behave according to their specialization in each geographical locality. Thus, any study dealing with this pathogen should clearly indicate its origin.

Many modes of survival of *X. campestris* pv. *vesicatoria* have been suggested, *e.g.*, overwintering in dead tomato debris and diseased leaves[12,16,18], the rhizosphere of wheat, tomato and soybean[7], tomato seedlings[13,14], symptomless tomato plants[5,15], seeds[10,13,16,22] and soil[18]. In previous studies with *X. campestris* pv. *vesicatoria* it was demonstrated that diseased leaves had an

endophytic population of this bacterium[2,20]. However, there is no comprehensive study dealing with one isolate and one type of plant in different surviving environments.

The purpose of this work was to study the survival of an isolate of *X. campestris* pv. *vesicatoria*[6] and to assess its potential as a source of inoculum in the next growing season.

Materials and methods

Twenty isolates of *X. campestris* pv. *vesicatoria* were isolated in the summer from infected pepper plants from the Yezreel Valley, the Lachish region, the Jordan Valley and the Jericho area. An isolate of *X. campestris* pv. *vesicatoria* from the Jericho area was used in most of the survival studies. The bacteria were isolated on Nutrient agar (Difco) plates supplemented with 0.15 g/l sodium deoxycholate (ND). The pathogens were kept on Nutrient agar (NA) slopes at room temperature (20–30°C) and transferred to fresh medium every week. Isolation of the pathogen from roots and rhizosphere was carried out on diagnostic medium containing (g/l) glucose, 5; peptone, 5; DL-methionine, 0.1; cycloheximide, 0.04; 1 : 1 complex of ferric-8-hydroxyquinoline, 0.0045; deoxycholic acid 0.15; agar, 17[18]. Induction of pigment formation was done in a medium containing (g/l) (SPY) sucrose, 10; peptone, 10; yeast extract, 3; $MgSO_4$, 1.5; NaCl, 5; $CaCl_2$, 1; K_2HPO_4, 1.5. To prevent loss of pathogenicity, the pepper leaves were inoculated with the pathogen and the bacteria were reisolated at least once a month, according to the leaf enrichment method[21]. Pepper seeds (*Capsicum annuum*) cvs. 'Maor, Californi, Zohar, Zahov-Naharia' and seeds of wheat (*Triticum durum*), sorghum (*Sorghum vulgare*), cucumber (*Cucumis sativus*), bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) and tomato (*Lycopersicon esculentum*) were used. They were obtained from 'Hazera' Co., Haifa.

The experiments were carried out in a fully controlled environment growth chamber (30 ± 2°C, 16 h light 75 W/m^2, 8 h darkness) or in a mist Chamber (30 ± 2°C, daylight, 5 sec mist every 30 min). Experiments were conducted 2–3 times in a completely randomized fashion in 10 replicates.

Growth conditions, inoculum preparation, inoculum procedures, seeds and soil infestation (one per cent of bacterial cells initially present at inoculation time survive on the dried seeds[21]) and pathogenicity tests were as described elsewhere[14].

Sandy loam soil of Rehovot maintained at near field capacity was infested by adding bacterial suspension at a final concentration of 10^8 CFU/g soil. Field soil was infested by spraying 51 of 10^8 CFU/ml of bacterial suspension on area of 800 m^2. After infestation, plants were further wetted by drip irrigation, with no unnecessary wetting of the foliage.

The disease index was estimated using an index of 0–3 (0 = no symptoms, 3 = 10 or more scabs per leaf, using the four mature upper leaves of each plant). Highly infested pepper leaves (disease index = 3.0) were dried by one of the following methods: a) Leaves were dried at 35°C for 10 days and milled; b) Leaves were frozen at −80°C for 24 h, lyophilized to dryness, and milled. Infested pepper leaves were kept in hermetically closed glass boxes containing silica gel at the bottom to prevent condensation on the walls of the glass boxes, at 4°C for several months.

Bacterial presence in seeds, soil and roots was detected by the following methods: a) infested seeds, soil and roots were soaked for 2 h in saline (8.5 g NaCl/l) and bacteria in the suspension were counted using the ten-fold dilution method on ND medium after incubation for 48 h at 30°C; b) the saline suspension was placed on leaves using the enrichment technique[21]. Symptoms were recorded 9 days after inoculation, and the pathogen was reisolated in ND medium; c) seeds were germinated in a growth chamber. At the cotyledon stage they were transferred to mist chamber for 9 days, then disease symptoms were recorded; d) seed samples (1000 seeds each) or 1 g soil were soaked in ND liquid medium at 28°C and vigorously shaken for 4 h. Viable bacterial counts from the suspensions were estimated by spreading 0.1 ml of a 10-fold dilution with a glass rod on the same medium supplemented with 2% agar. Bacterial counts from roots were made by the same procedure[1] on the

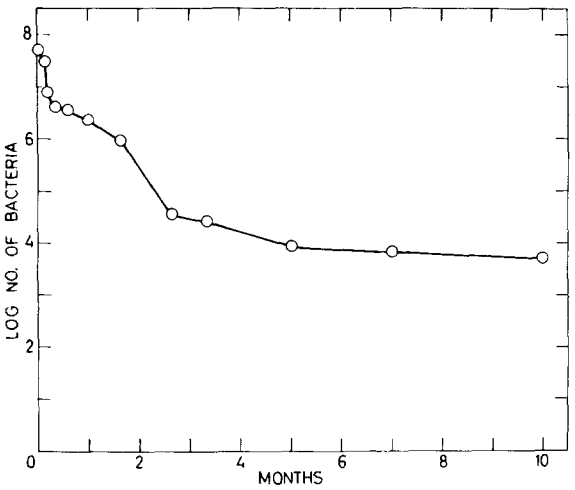

Fig. 1. Survival of *Xanthomonas campestris* pv. *vesicatoria* on artificially infested pepper seeds.

selective medium; 3) suspected colonies which developed on ND medium were grown for 96 h in SPY medium at 30°C and their carotenoids were extracted according to the methods of Starr and Stephens[24] or Nur *et al.*[17] and compared to a known isolate of *X. campestris* pv. *vesicatoria.*

Determination of endophytic bacteria was done by obtaining triplicate samples, each of 5 g of leaves and surface disinfested by immersion in 3% NaOCl for 5 min. The sterilized samples were washed with sterile water to remove traces of hypochlorite and homogenized in 20 ml of sterile saline in an Omni-mixer (Sorvall) maintained in an ice bath. The homogenate was diluted serially 10-fold and 0.1 ml portions were spread with a glass rod on the surface of Nutrient agar (Difco) plated. The inoculated plates were incubated for 3 days at 30 ± 2°C.

To test the efficiency of the leaf disinfection method, disinfested leaves were placed on the same agar as above for 5 h and then removed. No pathogen colonies developed after 48-h incubation.

Results

Survival of X. campestris *pv.* vesicatoria *on artificially infested pepper seeds*

Survival of *X. campestris* pv. *vesicatoria* was followed on 5 lots of infested pepper seeds (50 g each, inoculation level of 10^8 CFU/ml). Suspected isolated colonies were tested for pathogenicity. Three seed lots soaked in sterile distilled water were used as controls.

It was found (Fig. 1) that the pathogen had the ability to survive on infested pepper seeds. Bacterial numbers decreased with time. After 5 months, the numbers of the pathogen reached a steady level. The pathogen present on the seeds maintained its virulence towards the pepper plants throughout the experiment.

Development of symptomless plants originating from infested seeds

Pepper seeds were infested with various bacterial suspensions ranging from 10^3 to 10^9 CFU/ml (triplicates, 20 seeds/pot at each inoculum level). At the age of

2 true leaves, plants were transferred to a mist chamber. Control seeds were soaked in sterile water, or grown in a growth chamber under R.H. $<40\%$, which prevented bacterial multiplication on the leaf surface. After one month, only 10–15% of the plants showed limited scab symptoms after all treatments. But, it was found that a large latent population of *X. campestris* pv. *vesicatoria* (ranging from $2 \cdot 10^6$ to 1.1×10^7 pathogens/g leaf) existed inside the leaves, but not on their surfaces, in the apparently symptomless plants. All pathogenicity tests of these isolates were positive. No pathogens could be detected in plants grown under R.H. $<40\%$. In addition, no relationship could be found between the inoculation level of the seeds and the level of *X. campestris* pv. *vesicatoria* inside the pepper leaves.

In an additional trial, 16 samples (100 g each) were collected from different seed production fields from all over Israel. No scab symptoms were observed in the parental plants. Ten samples were found, by the leaf enrichment procedure, to be contaminated with virulent isolates. These seeds were germinated in the greenhouse and, at the age of 2 true leaves, they were transferred to the mist chamber for 4 weeks. No visible symptoms developed in the most. However, the endophytic pathogenic population of *X. campestris* pv. *vesicatoria* was found to be 10^5–10^6 CFU/g leaf. Later, these seedlings were transferred to the field for 2 months. No symptoms appeared after this period of time. However, the internal pathogenic bacterial population kept its high level throughout the growing season.

Determination of X. campestris *pv.* vesicatoria *in the stem and pepper rhizosphere*

Pepper seeds were infested with 10^7 CFU/ml (triplicates, 10 plants/replica), germinated and transferred to the mist chamber. After 14 days the presence of *X. campestris* pv. *vesicatoria* was followed separately inside the stem and in the

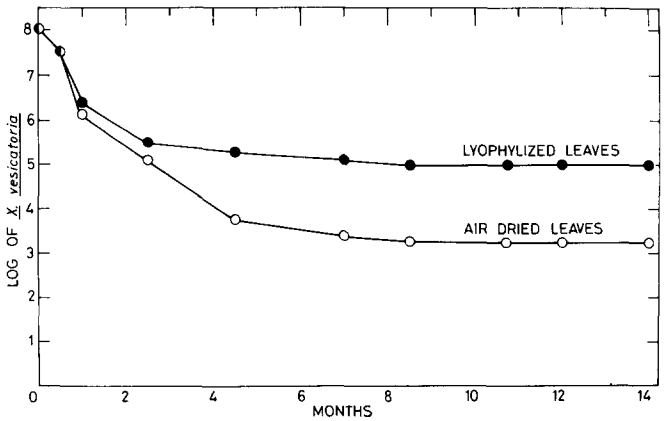

Fig. 2. Survival of *Xanthomonas campestris* pv. *vesicatoria* in dried pepper leaves.

Table 1. Survival of *Xanthomonas campestris* pv. *vesicatoria* on non-host seeds, leaves and rhizosphere

Plant species	No. bacteria/g							
	Seeds						Rhizosphere	Leaves
	Days							
	7	14	30	50	80	100	30	30
Sorghum bicolor	3×10^5	1.5×10^3	0	0	0	0	3×10^4	0
Cucumis sativus	3.8×10^5	2×10^3	9×10^1	0	0	0	6×10^4	0
Phaseolus vulgaris	1.1×10^5	3.5×10^4	0	0	0	0	6×10^4	0
Pisum sativum	2×10^5	2×10^2	0	0	0	0	2.5×10^4	0
Triticum durum	1.1×10^5	2.4×10^2	1×10^2	0	0	0	8×10^4	0
Lycopersicon esculentum	8×10^6	4.2×10^6	1.2×10^5	1×10^4	3×10^3	1×10^3	5×10^4	0

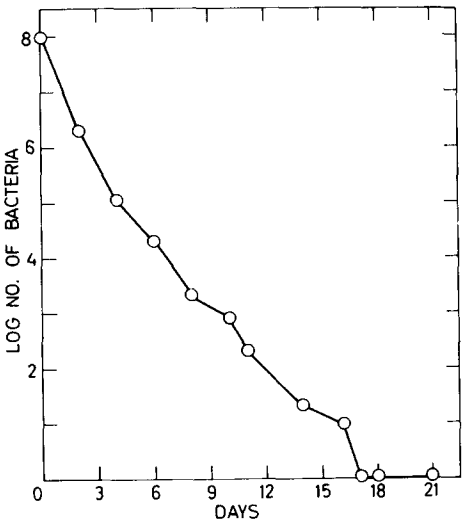

Fig. 3. Survival of *Xanthomonas campestris* pv. *vesicatoria* in sandy loam soil.

rhizosphere. The pathogen could not be isolated from the stem, but a large population (10^6 CFU/g roots) of *X. campestris* pv. *vesicatoria* was present in the rhizosphere of pepper.

Survival of X. campestris *pv.* vesicatoria *on seed, leaves and in the rhizosphere of non-host plants*

Seeds of sorghum, pea, cucumber, bean, wheat and tomato were infested with *X. campestris* pv. *vesicatoria* (10 g of each species, triplicates, inoculum level of 10^7 CFU/ml) and dried. The number of *X. campestris* pv. *vesicatoria* cells was estimated after several periods of time. Triplicates (100 seed/replica) were sown in sandy loam soil. After germination, seedlings were transferred to a mist chamber for 14 days. Then number of *X. campestris* pv. *vesicatoria* in the leaves and in the plant rhizosphere were recorded. It was found (Table 1) that the pathogen survived on tomato seeds for at least 100 days, whereas it disappeared from other non host seeds after only 30 days. In the rhizosphere the pathogen survived at the same level on all plant roots tested but could not produce symptoms in the non-host leaves incubated under mist.

Survival of X. campestris pv. vesicatoria *in dried pepper leaves*

Five lots of highly infected pepper leaves (10 g dry weight/lot, disease index = 3.0) were dried at 30°C and 5 similar lots were lyophilized to dryness. Samples (0.25 g each in triplicates) were tested periodically for the presence of *X. campestris* pv. *vesicatoria*. It was observed (Fig. 2) that the pathogen can survive and retain virulence for long periods of time, in dried pepper tissues, and after 4 months the pathogen reached a steady level (10^4 CFU/g dried leaf).

Survival of X. campestris *pv.* vesicatoria *in sandy loam soil*

Twelve samples (triplicates, 20 g soil/replica) of sandy loam soil of Rehovot were infested and maintained in polyethylene bags at 25 ± 3°C. After various periods of time, *X. campestris* pv. *vesicatoria* was isolated from the soil and tested for pathogenicity. It was found (Fig. 3) that the pathogen can survive in sandy soil for only 16 days and that it cannot be detected after that.

Overwintering of X. campestris *pv.* vesicatoria *in Yezreel Valley*

Three pepper seed lots were infested by either 10^7 or 10^9 CFU/ml and dried. Samples (50 g each) were buried (5 cm depth) in hermetically sealed glass boxes in the field for 1.5 year. Local soil samples (200 g each, triplicates) infested with 10^9 CFU/g soil (final concentration) were also buried. The surface of the soil of a pepper field was infested with the pathogen. During the burial period the boxes and the soil were subjected to temperatures ranging from 0.3°C to 29.8°C (31 days below 5°C) in the winter and from 7.9°C to 42.2°C in the summer (122 days above 30°C). The infested field experienced 84 rain days (above 1 mm a day) and total rain of 573 mm, only in the winter. In the summer the field was sprinkle-irrigated to near field capacity. The crops growing after pepper in the field were winter wheat and early spring industrial tomatoes (non-hosts to the pathogen). After 18 months, the bacteria in buried seeds and soil and in the soil from the field were counted.

It was found (Table 2) that the pathogen can survive at least 18 months under the above conditions either in soil or in seeds, but its population decreases markedly (10^1–10^2 CFU/g sample). Extraction of the typical carotenoid of *X. campestris* pv. *vesicatoria* (Fig. 4a, b) from suspected colonies and pathogenicity tests demonstrated the identity of the isolates.

Effect of source of inoculum and its application in the soil on X. campestris *pv.* vesicatoria *development in the leaves*

Highly infected pepper leaves (disease index = 3.0) were placed on the soil surface in pots containing pepper plants (5 true leaves) or to pepper nursery pots

Table 2. Overwintering of *Xanthomonas campestris* pv. *vesicatoria* in seeds and soil

Source	Inoculation level CFU/ml	No. of bacteria in one-g sample on NA medium*	No. of bacteria in one-g sample on ND medium*
Seeds	10^7	4×10^2	3×10^2
Seeds	10^9	1.5×10^2	1.6×10^2
Soil	10^9	2×10^1	3×10^1
Field soil	10^8	1×10^1	1.1×10^1

* Means of six replicates, counted after 18 months.

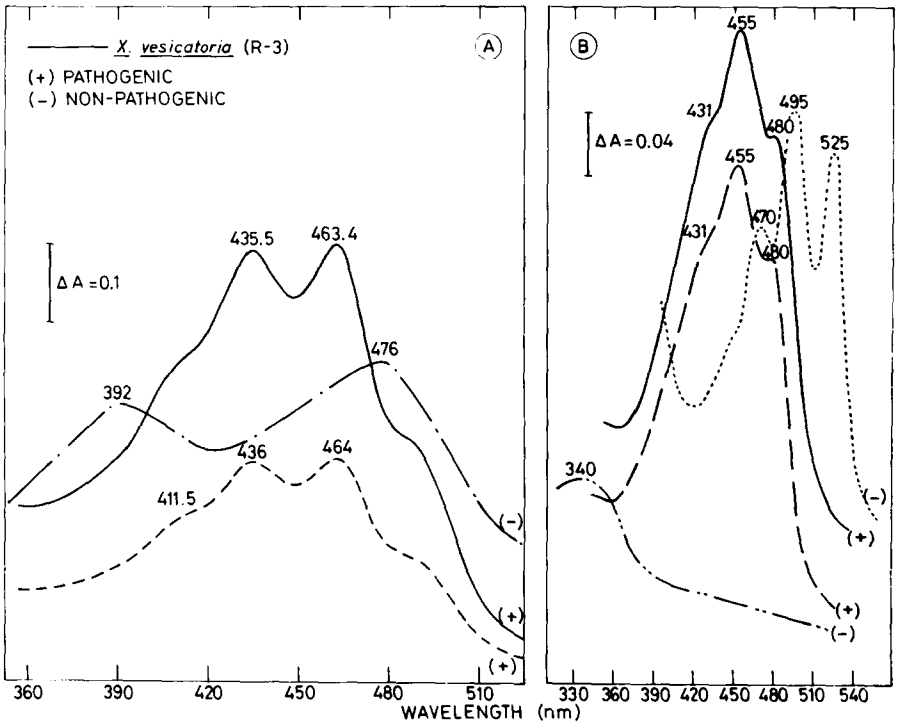

Fig. 4. Absorption spectra of carotenoids from suspected bacterial colonies obtained from soil or seeds. **A.** Spectra of partially purified carotenoids. **B.** Spectra of crude carotenoids extracted in methanol : chloroform : water.

(3 diseased leaves per pot, 10 replicates each). Half of the leaves were covered with a 2 cm layer of sterile vermiculite. The same procedure was carried out with a bacterial suspension (10^8 CFU/ml, 100 ml/pot). All pots were then transferred to a mist chamber. Pots inoculated with sterile water or healthy pepper leaves were used as controls. After one month in the mist chamber, the endophytic population of *X. campestris* pv. *vesicatoria* and symptom appearance was recorded.

No symptoms appeared in plant leaves, but plants (nursery or mature) had a massive endophytic bacterial population (ranging from 1.8×10^4 to 4.8×10^5 CFU/g leaf). No difference in these bacterial populations was observed when the origin of inoculum was from buried or surface diseased leaves. All tested isolates were virulant when inoculated on to pepper plants by the conventional inoculation procedure[3,5]. Thus, the disease can pass from diseased plant to healthy plant and leaves are a source of inoculum. However, disease could not originate from soil infested with bacterial suspensions under the conditions tested.

Discussion

This study has demonstrated that isolates of *X. campestris* pv. *vesicatoria* that infect pepper are capable of multiplying endophytically in pepper leaves that do not show any scab symptoms. In addition, commercial seeds obtained from apparently healthy plants and fruits contained the pathogen. This kind of phenomenon has also been observed in tomato leaves inoculated with the pathogen[14]. Epiphytic and endophytic pathogenic bacterial populations are important in transferring the disease within the growing season. Our results indicate that this pathogen has a resident phase on pepper leaves just as it has on tomato plants[13]. Such a type of life phase may explain the sporadic appearance of the disease in Israeli fields, *e.g.*, slight infection in some growing seasons and heavy epidemics in others, probably depending on environmental factors.

It has been suggested that some isolates of *X. campestris* pv. *vesicatoria* could not survive for more than 2 weeks in natural soil and 2 months in sterile soil[18]. We have shown that the populations of the isolates tested markedly decreased with time in soil. Nevertheless, it was possible to find surviving cells in the field the next season. Thus they were a potential source of inoculum in following pepper crops. In addition, survival of pathogenic bacteria in non-host rhizosphere is very important for overwintering of the pathogens[19]. *X. campestris* pv. *vesicatoria* overwinters in the rhizosphere of wheat, tomato and soybean[7]. Our work has confirmed those findings. However, the pathogen could not be established in the non-host foliage, but only in pepper foliage. Furthermore, we have found that diseased leaves buried in the soil or placed on the soil surface were a source of inoculum for pepper seedlings or for plants grown in the infested soil. It seemed that the pathogen reached the upper leaves, multiplied latently and later initiated disease.

The great versatility of the means by which *X. campestris* pv. *vesicatoria* survives from one season to the other and within the same season, ensures possible disease outbreaks under normal environmental conditions, at any time and place in pepper production fields.

Seed treatment, crop rotation, *etc.*, may decrease the number of inoculum sources and the inoculum size. But to fully prevent outbreaks of the disease appears to be a very difficult task.

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