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INFECTION STUDIES OF *PSEUDOMONAS TOMATO*, CAUSAL AGENT OF BACTERIAL SPECK OF TOMATO

By

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Pseudomonas tomato, the causal agent of bacterial speck of tomato (*Lycopersicon esculentum*), was isolated on King B medium supplemented with penicillin and cycloheximide. An inoculum concentration of 10^4-10^5 colony-forming units per ml was used to obtain typical symptoms. Wounding of leaves by rubbing them with carborundum powder, or spraying with diluted wax solvent, significantly increased disease incidence. The best inoculation was obtained by subjecting the plants to 40°C for 30 min to 3 h or by incubating them for 24 h under mist prior to inoculation. The physiological age of the plants did not affect disease development. The bacteria survived in and were reisolated from soils, commercial seeds and plants after periods varying from 2 months to one year.
KEY WORDS: Lycopersicon esculentum; epidemiology.

INTRODUCTION

Bacterial speck of tomato was first reported by Okabe (10). The disease is caused by *Pseudomonas tomato*, included in Bergey's Manual of Determinative Bacteriology (3) in the group of bacteria related to *P. syringae*. Typical symptoms on tomato leaves and fruits are 1-3 mm black lesions with a yellow, chlorotic halo (17).

The disease was first observed in Israel in 1970 (16) and since then has been spreading in tomato fields throughout the country. The degree of damage and the epidemiology of the disease in Israel are not known.

Bacterial speck can cause complete loss of the crop if the attack comes early in the seedling stage; up to 12% loss in yield of tomatoes may occur in less severely attacked fields (12).

We have studied mechanical, chemical and environmental factors affecting infection by *P. tomato* under controlled conditions and the survival of the pathogen in soils, seeds and contaminated plants.

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MATERIALS AND METHODS

Plant material and growth conditions

Tomato (*Lycopersicon esculentum*), cv. VF 198, was used as a host plant; this cultivar is widely used in Israel. In a preliminary survey of different cultivars of tomato, highest susceptibility to infection by *P. tomato* was obtained in cultivar VF 198 when compared with Mecheast 5S, VF 134-1, VF 145-513 and VF 145B-7879. Seeds were obtained from Hazera Co., Haifa, Israel. The seeds were germinated in sterile peat and vermiculite 50:50 (v/v) in a greenhouse maintained at 25°C. Seedlings were transferred at the age of two true leaves to plastic pots, 10 cm wide at the base and 14 cm in height, containing 600 g sterile sand. Plants were inoculated only after the development of the third leaf. After inoculation, the plants were generally grown under a misting device at 25 ± 2°C, with a 12 h light–12 h dark regime. The plants were irrigated every week with 10 ml of a 1.0% (w/v) commercial fertilizer solution (20:20:20, NPK).

Organisms and growth media

An isolate of *P. tomato* from the collection of the Division of Plant Pathology, The Volcani Center, Bet Dagan, Israel, was used in all experiments. Saprophytic *P. fluorescens* and *P. aeruginosa* were obtained from the collection of our department.

Stock cultures were kept at room temperature on nutrient agar (Difco) slants and transferred to fresh medium every month. Cultures used as inoculum were grown on a yeast-peptone (Difco) liquid medium, containing 0.06 M potassium phosphate buffer, pH 6.8, in a shaking bath at 30°C for 12-18 h. The cultures were centrifuged at 12,000 *g* for 7 min and were resuspended in saline (0.85% w/v NaCl) to give 0.1 absorbance units at 560 nm in a Junior II Coleman spectrophotometer, corresponding to about 10^7 colony-forming units (CFU)/ml.

Bacteria were counted by the dilution plate count method (12). Colony counts were made in plates with fewer than 400 colonies per plate.

Isolation procedure

Pseudomonas tomato was isolated in a King B medium (7), containing 200 units of penicillin (Teva Ltd.) and 2 μg cycloheximide (Sigma) per 10 ml of medium. In this medium Gram-positive bacteria and fungi did not develop, enabling clear distinction of suspected fluorescent colonies. Isolates were tested for pathogenicity on tomato plants.

Attempts were made to isolate *P. tomato* from soils, the surface of tomato seeds, inside tomato seeds, leaves, stems and vascular system of inoculated plants, and typical lesions of bacterial speck. A 1-g sample of soil, seed or leaf was taken aseptically and shaken in 100 ml sterile saline for 1 h. The suspension was filtered through sterile No.1 Whatman filter paper to remove large particles. Tenfold dilutions were then filtered through sterile 0.45 μm Millipore filters, which were placed on the medium. Fluorescent colonies were counted after 24 h incubation at 30°C and tested for pathogenicity.

To check for contamination inside the seeds or vascular system, seeds or pieces of stems were surface sterilized with 70% ethanol followed by 1.0% sodium hypochlorite, each for 30 sec under vacuum. The vacuum was released abruptly to favor sterilization of the seed or stem surface. The samples were washed five times with sterile water

to remove traces of ethanol and hypochlorite, and then homogenized for 1 min in sterile saline in a Waring Blendor; bacteria were counted by the pour plate method.

Inoculation procedures

Before inoculation, plants were incubated under mist at 25°C for 24 h, or under normal atmospheric conditions at 40°C for periods of 30 min–3 h. Under these conditions the plants did not wilt. Other pretreatments included incubation at 6°C for 12 h, or leaving the plants without water until they started to wilt. Also, plants were wounded with 300 grid carborundum powder, punctured ten times per leaflet with a sterile needle, or sprayed to runoff with the following diluted wax solvents (0.1% in 70% ethanol): ether, petrol ether, chloroform, benzene, or 0.001 N NaOH or KOH (in water). The plants were inoculated by spraying until runoff with suspensions of *P. tomato* containing 10^1-10^7 CFU/ml, or by slowly injecting into the stem tissue 1-ml samples of the bacterial suspensions. To achieve a slow release of the suspension into the tissue, a soft rubber tube (3 mm internal diameter, 20 cm length) sealed at one end and attached to a hypodermic needle (27G x 1″) at the other end (separated by a clip), was filled with the bacterial supension under pressure, as indicated by the swelling of the tube. After insertion of the hypodermic needle into the stem, the clip was removed, thus allowing the suspension to diffuse slowly into the stem for about one hour.

Scanning electron microscopy (SEM)

Pieces of leaves 0.5 cm^2 were fixed for 5 h in a 50% (in water) glutaraldehyde solution and then washed with acetone solutions of 50% for ½ hour, 70% for ½ h, 100% for ½ h, and 100% for 1 h. This method has been recommended for plant surface fixation without damage or change in cell morphology (5). The samples were dried in a critical point dryer (Druva) in a CO_2 environment. The dry samples were placed on stubs, covered with gold, and examined under a Cambridge-4 stereoscan microscope.

RESULTS

Factors affecting disease

The effect of *P. tomato* inoculum concentration on infection level in tomato plants is summarized in Table 1. Plants wounded with carborundum powder before inoculation did not develop disease symptoms when inoculated with bacterial suspensions containing fewer than 10^4 CFU/ml. Typical symptoms, similar in severity to those observed under field conditions (5-30 speck lesions per leaflet), were observed at inoculum concentrations of $10^4 - 10^5$ CFU/ml; above this, atypical symptoms such as falling leaves, yellow leaves and blackening of growing tips were observed. Disease developed mainly in plants incubated under mist after inoculation. Very few symptoms were observed in plants incubated under normal greenhouse conditions.

TABLE 1

EFFECT OF INOCULUM CONCENTRATION OF *PSEUDOMONAS TOMATO* ON BACTERIAL SPECK SYMPTOMS IN TOMATO

Tomato leaves were wounded with carborundum powder and sprayed with suspensions of bacteria. Plants were kept under mist. Lesions were counted after 7 days, on all leaflets of 20 plants per treatment. Experiment was done three times.

Inoculum concentration (CFU/ml)	*Lesions/leaflet* (no.)	*Falling leaves* (%)	*Yellow leaves* (%)	*Black tips* (%)
0	0 d*	0–6.5	0–2.6	0
10^1–10^3	0 d	0–6.5	0–2.6	0
10^4	1.0 c	0–6.5	0–2.6	0
10^5	16.7 b	20	11	0
10^6	28.2 a	30	25.2	0
10^7	leaf completely covered	52	26.1	30

*Numbers followed by the same letter did not differ significantly at P = 0.05.

Pretreatment by wounding with carborundum, or by spraying with diluted wax solvents, significantly increased the number of lesions per leaflet (Table 2). No apparent damage of epidermal cells or opening of stomata on chemically treated leaves could be observed under SEM in ten different preparations of each treatment. Very few symptoms could be observed in plants inoculated with 10^5 CFU/ml and pretreated with water, 70% ethanol or by puncturing with a sterile needle. The greatest increase in disease level was obtained by preincubating plants before inoculation under mist for 24 h. Preincubation at 40°C for periods of 30 min to 3 h increased disease incidence to a lesser extent (Table 3). Low temperature (12 h at 6°C) or drying plants up to the beginning of wilt did not affect infection. A combination of treatments, such as mist for 24 h plus 40°C for 1 h, did not produce a synergistic effect (Table 3). All stomata observed under SEM in plants preincubated under mist or at 40°C for 3 h were open, to a greater or lesser extent. *Pseudomonas tomato* was capable of infecting tomato plants at different physiological ages, such as cotyledons, first true leaves and 3-9 leaves.

All attempts to obtain disease symptoms by introducing bacteria directly into the stems and leaves, or through wounded roots soaked in *P. tomato* suspensions, failed. Also, no spread of disease was observed when infected leaves were removed and the plants were kept growing under mist, giving further support to the observation that *P. tomato* cannot infect through the vascular system.

TABLE 2

CT OF MECHANICAL AND CHEMICAL RETREATMENT ON SEVERITY OF ACTERIAL SPECK SYMPTOMS ON MATO LEAVES INOCULATED WITH *PSEUDOMONAS TOMATO*

were inoculated by spraying with a suspension CFU/ml, and incubated under mist. Lesions counted after 7 days on all leaflets of 16 per treatment. Plants pretreated and non- ted or inoculated with saprophytic *P. flu-* r were symptomless. The experiment was hree times.

Pretreatment	*Lesions/leaflet* (no.)
ol (water or 70% ethanol)	2.16 c**
undum	16.21 b
uring with needle*	2.96 c
oform, 0.1%	16.40 b
0.1%	15.60 b
ether, 0.1%	22.40 a
ne, 0.1%	15.10 b
l, 0.001 M	21.80 a
0.001 M	22.60 a

times per leaflet.
mbers followed by the same letter did not significantly at P = 0.05.

TABLE 3

EFFECT OF MOISTURE AND TEMPERATURE PRETREATMENT ON DISEASE SEVERITY OF TOMATO PLANTS INOCULATED WITH *PSEUDOMONAS TOMATO*

Plants were inoculated by spraying with a suspension of 10^5 CFU/ml, and incubated under mist. Lesions were counted after 7 days, on all leaflets of 10 plants per treatment. Pretreated plants inoculated with *P. aeruginosa* or *P. fluorescens* were symptomless. The experiment was done three times.

Pretreatment	*Lesions/leaflet* (no.)
Control	2.97 d*
Drying up to wilting point	2.52 d
6°C for 12 h	2.09 d
40°C for 0.5–3 h	13.1–16.0 b
24 h under mist	29.41 a
24 h under mist + 3 h 40°C	24.69 a
24 h under mist + 3 h dried	7.57 c

*Numbers followed by the same letter did not differ significantly at P = 0.05.

Survival of P. tomato

The presence of the pathogen in soil was investigated in three fields in the same area (Lakhish region, in southern Israel). *Pseudomonas tomato* was readily isolated from all fields with diseased plants. A high percentage of the fluorescent colonies isolated caused typical speck symptoms on tomato. After one year, samples were taken from the same fields (where wheat was then growing) and 8% of the sampled (3/40) fluorescent colonies isolated, caused disease.

Three lots of commercial seeds were tested for the presence of *P. tomato*. The batch used for this work (VF 198) was free of *P. tomato*, but two batches of seeds (VF 134 and Mecheast 5S) utilized for field crops which had severe infection, were contaminated with the bacterium. Tomato seedlings (100/1000 of Mecheast 5S and 950/1000 of VF 134) from contaminated seeds grown in sterile soil under mist had speck symptoms, whereas clean batches grown under the same conditions were symptomless. After surface-sterilizing contaminated seeds and homogenizing them, *P.*

tomato could not be reisolated; furthermore, plants growing from surface-sterilized seeds were symptomless.

The presence of *P. tomato* in new leaves of inoculated plants was followed for a period of 2 months. The number of bacteria per mg leaf (dry weight) was determined. Plants were spray-inoculated (10^5 CFU/ml) and grown under mist for 7 days until symptoms developed; then, half of the plants were kept under mist and the other half were transferred to greenhouse conditions. In new leaves of plants incubated under mist, pathogen counts increased and then declined during a period of 2 months, but were still relatively high (Table 4). Disease symptoms developed in new leaves. The pathogen declined very fast in plants incubated without mist. Forty days after inoculation it was not possible to isolate the organism from the new leaves (Table 4), and they were symptomless. In non-inoculated control plants incubated under both conditions, only a few non-pathogenic fluorescent colonies per mg leaf (dry weight) could be isolated (Table 4).

Repeated attempts to isolate the organism from the vascular system of infected plants have failed.

TABLE 4

EFFECT OF HUMIDITY ON THE PRESENCE OF *PSEUDOMONAS TOMATO* IN NEW LEAVES OF TOMATO PLANTS

Plants were preincubated under mist for 24 h, inoculated by spraying with a suspension of 10^7 CFU/ml, incubated for 7 days under mist, and then incubated for 2 months under mist or dry greenhouse conditions. Results are CFU obtained from 3 leaflets, taken at random at each sampling, from each of 15 plants in every treatment.

Incubation time (days)	*CFU/mg dry wt. of new leaves*			
	Mist		Dry	
	inoculated	non-inoculated	inoculated	non-inoculated
7	376*	1**	3***	0
25	1414	0	2	1**
40	180	2**	0	1**
60	40	0	0	0

*Twenty fluorescent colonies selected at random from each inoculated sample incubated under mist were pathogenic to tomato.
**Non-pathogenic fluorescent colonies.
***All colonies from inoculated plants grown under greenhouse conditions were pathogenic.

DISCUSSION

Bacterial speck of tomato has been reported in Israel mainly in plastic-covered plants of the winter and early spring crops. Under these conditions, infection is

favored by high temperatures – which may reach 35–40°C during a sunny day, and by free water – which accumulates on the leaves (12). In our studies we were able to isolate *P. tomato* from soil, seeds and plants, using a medium containing penicillin and cycloheximide. However, fluorescent pathogens could not be differentiated on this medium from fluorescent saprophytes, but differentiation was achieved by inoculating plants and observing typical symptoms.

For successful infection of tomato plants incubated under mist, and appearance of typical lesions, 10^4-10^5 CFU/ml inoculum were required. Similar bacterial concentrations are required for development of symptoms by other phytopathogenic bacteria causing leaf spot diseases (8, 9).

Pretreatment of tomato plants prior to inoculation, by wounding the leaves with carborundum powder, greatly increased infection by *P. tomato*. To some extent this effect is comparable to that of sand, wind, hail or rain storms, which favor leaf spot diseases caused by phytopathogenic bacteria under field conditions (6, 15). Quantification of this method, however, is extremely difficult since the degree of wounding leaves varies greatly and can not be replicated.

Generally bacteria are not able to penetrate through the wax layer covering most plants (6, 8). By spraying the leaves with solutions containing wax solvents which had no visible damaging effect to the plant, it was possible to increase markedly the infection of tomato leaves by *P. tomato*. Leaves treated with wax solvents and observed under SEM showed closed stomata. There is a possibility that the solvents dissolved the wax in some spots not observed under the SEM, thereby enabling direct contact between *P. tomato* and epidermal cells under conditions similar to those prevailing in the case of open stomata, thus favoring infection.

It is well known that bacteria causing leaf spots invade the plant mainly via the stomata (6, 8, 12). Preincubation of plants under mist for 24 h before inoculation – under which conditions stomata are fully open – increased disease to the highest level. Also pretreatment by heat – 39°C for 6 h as observed before (12), or 40°C for a period as short as 30 min – significantly increased disease, probably due to opened stomata as seen in this work in SEM preparations, and as found on tomato plants incubated at high temperatures (1). On the other hand, drought or cold treatments result in closing of stomata and a slow-down in bacterial development and movement (6, 8, 11), thus reducing infection. *Pseudomonas tomato* infected tomato plants at all stages of growth, provided plants were properly pretreated and incubated under optimal conditions for disease development, such as under mist before and after inoculation. In general, bacteria causing leaf spot diseases are not transferred nor do they move through the plant vascular system (6). Attempts to demonstrate infection of leaves by *P. tomato* located in the vascular system, were unsuccessful. Therefore, bacterial speck of tomato may be considered as a local-lesion, rather than a systemic disease.

Two months after inoculation, substantial numbers of the pathogen were found in new leaves of plants incubated under mist. This finding corroborates the work of Schneider and Grogan (8). In contrast to incubation under mist, few CFU of the pathogen were obtained in plants incubated under normal greenhouse conditions.

Forty days after inoculation it was not possible to reisolate *P. tomato* from new leaves. Examination at shorter intervals of incubation time revealed resident populations of *P. tomato* inside tomato leaf trichomes (13), which may serve as the main site for survival under dry conditions (13). We have observed that in an infected tomato field, after spells of dry weather characteristic of early spring in Israel, disease was arrested and plants recovered fully.

Schneider and Grogan (12) could not isolate the pathogen from commercial seed lots in more than 20 different tests conducted in California. We isolated *P. tomato* from two batches of commercial seeds that produced severely infected crops in the field. After surface sterilization, seeds were free of the pathogen and no diseased plants developed from the seeds upon incubation under mist. It is not known at what stage or how commercial seeds were surface contaminated.

It has been reported that *P. tomato* can survive, and the disease derive from inoculum in soil and plant debris (2, 4, 12). The pathogen was readily isolated in Israel from soils on which infected crops were growing, and from the same soils after one year of fallow. Schneider and Grogan (12) reported isolating the organism from soil in fields without a previous tomato crop, and concluded that *P. tomato* is an ubiquitous organism and a soil-borne plant pathogen.

It is not known how *P. tomato* was introduced into local fields. It may be spreading now by locally produced, improperly processed seeds.

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