

Distribution of Streptomycin-Resistant Strains of *Erwinia amylovora* in Israel and Occurrence of Blossom Blight in the Autumn

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Following failure in control of fire blight with streptomycin, the distribution of streptomycin-resistant strains of *Erwinia amylovora* in Israel was surveyed. During 1994–1997 109 pear, apple, loquat and quince orchards were monitored. Streptomycin-resistant strains of *E. amylovora* were recovered from flowers and from infected branches collected at 18 locations in the Sharon, Galilee and Golan Heights regions. In the Sharon region all the isolated strains of *E. amylovora* were streptomycin-resistant, whereas in the Galilee and Golan Heights, resistant as well as sensitive *E. amylovora* strains were recovered at different locations. In the southern coastal plain no resistance could be detected. Streptomycin-resistant strains of *E. amylovora* did not hybridize with the DNA probe SMP3, and resistance could not be transferred by mating to a sensitive strain, suggesting that streptomycin resistance in Israel is not plasmid-mediated. Fire blight symptoms were observed, for the first time, on pear blossoms during the autumn of 1994. A high population of 2×10^6 – 6×10^7 CFU/flower in the autumn of 1995 and of 1996 was correlated with the appearance of blossom blight symptoms. KEY WORDS: *Erwinia amylovora*; fire blight; streptomycin resistance.

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora*, was first detected in Israel on pear trees in 1985 (1,15,20). Since then, the disease has been observed in all pear-growing areas of the country, in apple and quince orchards (16) and in 1994 also on loquat (19). The population of *E. amylovora* in Israel was found to be homogeneous, although it had originated from several hosts and different regions (9). Control measures against fire blight disease include antibiotic sprays, with streptomycin being the preferred bactericide in North America. However, resistant strains of *E. amylovora* have been reported in many regions of the United States (3,6,8,14) and also in New Zealand (17). In Israel, streptomycin has been used for controlling the disease since 1986. Streptomycin-resistant *E. amylovora* strains were first detected in 1991 in an isolated pear orchard in the northern Negev. During the spring of 1995 a severe fire blight epidemic occurred in the Sharon and Galilee regions, which are the main growing areas of pear, apple and loquat in Israel. Failure of streptomycin to control the disease in most of the pear orchards

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in these regions suggested that resistant *E. amylovora* strains were already present. No comprehensive studies of the distribution of streptomycin resistance had been performed in Israel before 1994.

The most common pear cultivars grown in Israel are 'Spadona', 'Costia' and 'Gentile'. In spring, Gentile is the earliest to bloom (mid March), followed by Spadona and later Costia (in early April). In some years there is a second bloom period in Spadona and Costia during September–November, which differs from the late spring rat-tail bloom. This autumn bloom occurs primarily in orchards under stress, mainly because of insufficient irrigation during the late summer after fruit harvest. The autumn bloom is not uniform, as is the spring bloom, and it is dependent on rainfall in October–November. It differs in intensity from orchard to orchard and even within the same orchard trees may be in different blossom stages. Although streptomycin is not applied to pear orchards during autumn, it is applied to loquat orchards, since blooming in loquat occurs during November–December. It is noteworthy that in the Sharon region, orchards of loquat, pear and apple are adjacent to one another. The occurrence of fire blight on blossoms produced in the autumn was not recorded previously, nor was it known whether these flowers could support an epiphytic population of *E. amylovora*. The potential of the autumn blossom to serve as a source of inoculum for the spring blooming period is not known. In the present study we investigated the distribution of streptomycin resistance in Israel and determined the mechanism of resistance. In addition, we studied the presence of *E. amylovora* and its sensitivity to streptomycin on flowers during autumn. Preliminary results of this study were published elsewhere (10).

MATERIALS AND METHODS

Isolation of bacteria. Samples were collected from 45 orchards of pear, apple, quince and loquat during 1994–1995 and from 64 orchards during 1996–1997. Isolations were made from symptomless flowers and/or from infected tissues. From each orchard, three replicas of 25 flowers were collected at random. Each sample was suspended in 40 ml of sterile distilled water and serial tenfold dilutions were plated on CCT agar medium (7) with and without 100 $\mu\text{g/ml}$ of streptomycin sulfate. Plates were incubated for 3 days at 28°C. In orchards with fire blight symptoms, isolations were made also from infected tissues: flowers, shoots and branches with visible cankers. Small tissue pieces were ground in 5 ml sterile water with a homogenizer (Pro200, Pro Scientific Inc., Monroe, CT, USA) and 30 μl of homogenate was streaked on CCT and on CCT + streptomycin. At least five samples were tested from each orchard.

Identification of *E. amylovora*. Characteristic colonies of *E. amylovora* (i.e., light purple, opalescent color with a faint purplish center) that grew on CCT and CCT + 100 $\mu\text{g/ml}$ streptomycin were subcultured on Luria-Bertani (LB) medium (Difco) and on LB amended with 100, 500 and 1000 $\mu\text{g/ml}$ streptomycin, respectively. Isolated bacteria were tested for pathogenicity on slices of immature pears (13) and were identified by PCR using the primers and amplification conditions described by Bereswill *et al.* (2). Three characteristic colonies of *E. amylovora* obtained from each isolation were subjected to both tests. Positive-reacting strains were stored at –80°C in 20% glycerol.

Colony hybridization and bacterial conjugation. One hundred streptomycin-resistant *E. amylovora* strains, isolated from hosts and different orchards, were subjected to colony hybridization with the DNA probe SMP3 (12). Colonies were grown on Hybond N nylon membranes (Amersham Pharmacia Biotech, Little Chalfont, UK) overlaid on LB + streptomycin agar plates and incubated at 28°C for 48 h. The colonies were then lysed and fixed to the membrane in a microwave oven for 30 sec at 650 W. The probe, 0.5-kb *Bam*HI-*Ava*I fragment (12), was labeled with digoxigenin-11-dUTP using a labeling and detection kit (Boehringer Mannheim Corp., Mannheim, Germany). Prehybridization, hybridization and chemiluminescent detection of the probe were performed according to the manufacturer's instructions. *Escherichia coli* (DH5 α) containing the plasmid pCPP505 (12) was used as a positive control.

Bacterial conjugations were carried out with donor strains EaZ1 and Ea29-3, isolated in Israel from pear and loquat, respectively. Strain Ea1327, isolated from pear and containing resistance to rifampicin, was used as the recipient. Donor and recipient strains were grown for 16 h at 25°C on a rotary shaker in 5 ml LB medium amended with 100 μ g streptomycin (donor strains) or 150 μ g/ml rifampicin (recipient strain). The cultures of the donors and the recipient were mixed in a ratio of 1:1 and incubated for 15 min at room temperature, after which 10- μ l drops were plated on LB medium and incubated for 24 or 48 h at 25°C. Cell mixtures were suspended in 5 ml saline solution and serial tenfold dilutions were plated on LB medium amended with 100 μ g of streptomycin and 150 μ g of rifampicin. To determine the frequency of spontaneous resistant mutants, donor and recipient strains were plated on LB medium amended with rifampicin and streptomycin, respectively.

RESULTS

Distribution of streptomycin resistance. Resistant strains of *E. amylovora* were isolated from 18 locations in the Sharon, Galilee and Golan Heights regions (Fig. 1). All the streptomycin-resistant strains could also grow on high concentration of streptomycin (1000 μ g/ml). In Galilee and the Golan Heights, resistance was identified in 11 locations out of 17 that were surveyed (Fig. 1, Table 1). All the strains isolated from orchards of pear, apple and loquat in the Sharon region were resistant. All strains of *E. amylovora* isolated from pear, apple and quince orchards in the southern coastal plain and from a heavily infected orchard in the Jezre'el Valley were sensitive to streptomycin (Fig. 1, Table 1). In the northern Negev, resistance was detected only in one orchard during 1991, and no fire blight has occurred in this region since then. At all 18 locations where resistance was discovered, the frequency of streptomycin-resistant strains was 100%. The number of colonies recovered on plates of CCT and CCT + streptomycin was the same. At two locations (Giv'at Ada and Binyamina) in the Sharon region, the pear orchards have been heavily infected during four seasons since 1994; monitoring these orchards for streptomycin resistance during the four seasons revealed that the frequency of resistant strains was 100%.

Occurrence of blossom blight in autumn. Flowers were collected from 23 pear orchards (cv. Spadona) during autumn of 1995, and the population of *E. amylovora* was monitored in symptomless flowers. A high population of 10^6 - 5×10^7 CFU/flower was correlated with development of blossom blight in 16 orchards out of 23 (Table 2), whereas a lower

population of $1 \times 10^2 - 5 \times 10^2$ CFU/flower did not cause blight symptoms. During autumn of 1996, flowers were sampled from 35 orchards and examined for the presence of the pathogen. *E. amylovora* was detected in only ten orchards, and again blossom blight occurred only when a high population of bacteria was present, in six orchards out of ten (Table 2). The symptoms of fire blight observed during the autumn resembled those observed during the spring, namely, blackening of flowers and drops of ooze.

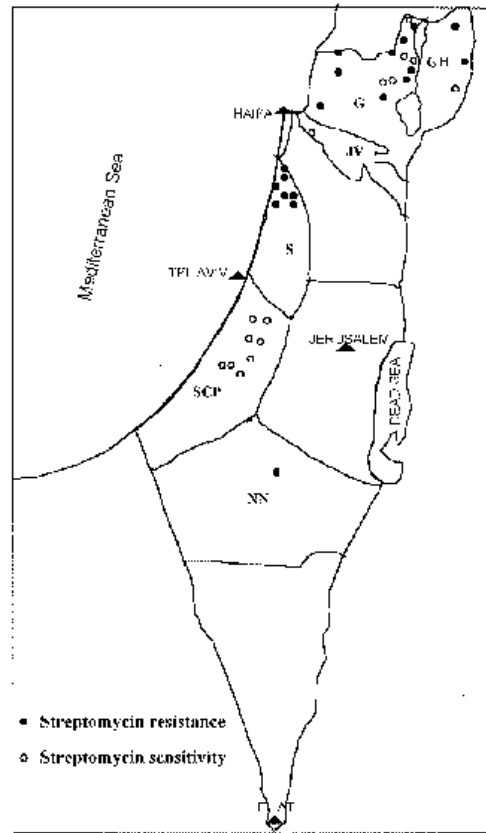


Fig. 1. Distribution of streptomycin-resistant strains in various regions of Israel during 1994–1997. GH- Golan Heights, G- Galilee, JV- Jezre'el Valley, S- Sharon, SCP- southern coastal plain, NN- northern Negev. Each dot represents a separate location.

During the autumn of 1995 and of 1996, the population of *E. amylovora* from symptomless flowers was also tested for streptomycin resistance. It was found that the presence of streptomycin-resistant strains was not correlated with the appearance of symptoms (Table 2) and could be found also from flowers with a low population of epiphytic *E. amylovora*. The streptomycin-resistant strains were detected in the autumn only from orchards in which streptomycin-resistant strains had been isolated in the previous spring but not from orchards with streptomycin-sensitive strains.

TABLE 1. Distribution of streptomycin resistance in Israel during 1994–1997

Region	Location	Host or Cultivar ^z	Resistance ^y
Golan Heights	Newe Ativ	Spadona, Spadochina	R
	En Ziwan	Spadona, Red Bartlett	R
Galilee	Qeshet	Jonathan	S
	Farod	Spadona, Costia, Gentile, Jonathan	S
	Sede Eli'ezer, Dishon	Spadona	S
	Metulla, Amirim	Spadona	S
	Mishmar haYarden	Spadona	R
	Adamit, Afeq, Ga'aton	Spadona	R
	Kefar Yuval, Yiftah	Spadona	R
	Rosh Pinna	Spadona, Jonathan	R
	Bar'am	Spadona, Jonathan, None-such	R
	Hazon	Spadona, Jonathan	R
Jezre'el Valley	Yagur	Spadona, Costia	S
Sharon	Zikhron Ya'aqov	Spadona, Costia	R
	Regavim, Shefiyya	Spadona	R
	Kefar Maharal	Spadona	R
	Bet Haniyya	Akko, Yehuda	R
	Binyamina	Spadona, Costia, Ein Shemer, Jonathan, Akko, Yehuda	R
	Giv'at Ada	Spadona, Costia, Jonathan, Akko, Yehuda	R
	Southern Coastal Plain	Qidron	Spadona, Costia, Gentile
Southern Coastal Plain	Orot	Spadona, quince	S
	Gedera	quince	S
	Arogot	Spadona	S
	Kefar Shemu'el	Spadona	S
	Be'er Toviyya	Anna	S
	Zerahya	Spadona, quince	S
	Gan Shelomo	Spadona, Costia, Spadochina, Anna, quince	S
	Northern Negev ^x	Revivim	Spadona

^zPear cultivars are Spadona, Costia, Gentile, Spadochina and Red Bartlett. Apple cultivars are Jonathan, None-such, Anna and Ein Shemer. Loquat cultivars are Akko and Yehuda.

^yThe presence of streptomycin-resistant and streptomycin-sensitive strains is indicated by R and S, respectively.

^xResistance was detected in 1991.

TABLE 2. Occurrence of blossom blight of pear and streptomycin resistance during the autumn of 1995 and of 1996

Location	Number of orchards	CFU/flower ^z	Blossom blight symptoms ^y	Streptomycin resistance ^x
<i>1995</i>				
Giv'at Ada	5	3×10^6 – 5×10^7	+	R
Giv'at Ada	1	2×10^2 – 3×10^2	–	R
Binyamina	4	4×10^6 – 5×10^7	+	R
Binyamina	1	2×10^2 – 3×10^2	–	R
Regavim	1	1×10^2 – 3×10^2	–	R
Yagur	2	4×10^6 – 2×10^7	+	S
Qidron	3	4×10^6 – 1×10^7	+	S
Gan Shelomo	2	2×10^6 – 2×10^7	+	S
Zerahya	1	4×10^2 – 5×10^2	–	S
Newe Ativ	3	2×10^2 – 3×10^2	–	R
<i>1996</i>				
Ga'aton	1	1×10^2 – 3×10^2	–	R
Binyamina	2	5×10^6 – 6×10^7	+	R
Givat Ada	2	2×10^2 – 3×10^2	–	R
Shefiyya	1	2×10^2 – 3×10^2	–	R
Gan Shelomo	2	7×10^5 – 9×10^6	+	S
Kefar Yuval	2	3×10^6 – 5×10^7	+	R

^zThe population of *Erwinia amylovora* was determined in symptomless flowers; 75 flowers were tested from each orchard. Numbers represent the lower and upper range of CFU/flower recovered on CCT and on CCT + streptomycin plates.

^y + indicates the presence and – the absence of blossom blight symptoms.

^xThe presence of streptomycin-resistant and streptomycin-sensitive strains is indicated by R and S, respectively.

Colony hybridization and bacterial conjugation studies. Colony hybridization with DNA probe SMP3 did not give a positive signal with 100 resistant strains that were tested. Positive results were obtained only with *E. coli* containing the plasmid pCPP505, which was used as a positive control (results not shown). In conjugation experiments, no transconjugants of the recipient strain (Ea1327) were obtained in matings with the donor strains (EaZ1, Ea29-3). The frequency of resistance of the recipient strain obtained from conjugations was as that of spontaneous mutations (*ca.* 2.0×10^9 per cell).

DISCUSSION

Streptomycin resistance was identified in the major pear, apple and loquat growing regions in Israel. In the Sharon region, where only resistant strains were isolated, severe fire blight has been recorded since 1994: most of the pear orchards were severely damaged, and many were uprooted. The prevalence of resistance in this region could result from the severe fire blight epidemic which has been continuously reported in all orchards throughout the years 1994–1997. Moreover, this region is also the main growing area for loquat, which was heavily infected by *E. amylovora* in the autumn of 1994. Since blooming of loquat occurs during autumn, it may provide a potential continuity of inoculum between seasons. In contrast to the Sharon region, the occurrence of resistance in the Golan Heights

and Galilee regions has not been detected at all locations. It is noteworthy that these two regions contain 70% of the pear and apple orchards in Israel but, unlike the Sharon region, fire blight did not occur every year. No correlation between the use of streptomycin and resistance could be found. Thus, in Farod (Table 1), where streptomycin has been sprayed every year, all the strains were sensitive, whereas in the Golan Heights, where streptomycin has not been applied regularly, resistance was identified at two locations (Newe Ativ and En Ziwan). In the southern coastal plain, growers applied several sprays of streptomycin per season but, so far, no resistant strains have been isolated from pear, apple or quince. Such a lack of correlation between streptomycin applications and resistance is consistent with previous reports from California (14) and Washington (8). The clustering of orchards harboring streptomycin-resistant strains in the Sharon and Galilee may suggest that resistance has developed independently in each of the two regions.

Two mechanisms of streptomycin resistance have been identified in *E. amylovora*: In Michigan (USA) it was mediated by aminoglycoside-modifying enzymes encoded by *strA* and *strB*, carried by the transferable plasmid pEa34 and Tn5393 (3,4,11); in the western United States, mutation has been more important in resistance development (5,14). Failure of the DNA probe (SMP3) to hybridize with colonies of Israeli resistant strains suggests that streptomycin resistance is not plasmid mediated. In addition, resistance could not be transferred by mating with a streptomycin-sensitive strain. These results indicate that mutation and not gene acquisition is the most likely mechanism for the development of streptomycin resistance in *E. amylovora* in Israel. The high level of resistance (1000 µg/ml) also supports the latter conclusion. The use of streptomycin in Israel is limited to fire blight management and involves several applications (3–5) during the blooming period in the spring, which lasts 3–4 weeks. The occurrence of resistance at a frequency of 100% in all cases might indicate that resistance had apparently developed from mutations exerted by intensive use at some locations, and which later became widespread.

Due to the wide spread of resistance to streptomycin in the population of *E. amylovora* in Israel, the recommendations for fire blight control were reevaluated. Since 1997 streptomycin has been removed from recommendations. Instead oxolinic acid 300 µg/ml (Starner 20% wp, Sumitomo Chemical Co., Japan) has been recommended exclusively for fire blight control, following field experiments in Greece (18) and in Israel (Shabi and Zilberstaine, unpublished results).

Disease symptoms during autumn were rarely seen prior to 1994. Although fire blight has been recorded in Israel since 1985, the autumn of 1994 was the first in which the disease was observed in many orchards. The autumn seasons of 1994 and 1995 were unusually warm and rainy. These conditions favor the development of symptoms, which were observed clearly in pear trees, where the population of the pathogen reached 2×10^6 – 6×10^7 CFU/flower. The present study provides the first report on the occurrence of blossom blight in autumn and the presence of an epiphytic population of *E. amylovora* in the autumn bloom. Furthermore, the epiphytic – albeit lower – population of *E. amylovora* in symptomless flowers during autumn may serve as a significant source of inoculum for the subsequent spring bloom. The loquat, which blooms during autumn, could serve as another source of inoculum if and when fire blight is not controlled. Streptomycin-resistant strains were isolated in autumn from loquat and symptomless pear flowers collected from orchards with or without apparent fire blight symptoms. The presence of resistant strains during autumn in pear and loquat orchards may form a continuous source of streptomycin resistance in the population of *E. amylovora* in Israel.

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