

SHORT COMMUNICATION
ONE YEAR SURVIVAL OF *ERWINIA AMYLOVORA* IN SYMPTOMLESS
PEAR SCIONS

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SUMMARY

Pear scions cv. 'Abate Fetel' on B29 rootstock were raised individually in pots from dormant buds. In autumn 1999, the main stems were divided into 5 groups and inoculated at 10 day intervals starting from 21 September with a virulent Rif^R mutant of *Erwinia amylovora*. For the inoculation, 30,000 cells of *E. amylovora* were deposited on a transverse cut 10 cm from the tip. After ensuring establishment of the pathogen, the five groups of plants were placed in the open and inspected weekly for the presence of fire blight symptoms. Scions treated with water were used as a control. All symptomatic scions were eliminated by mid-April 2000. The frequency of scions developing symptoms (21.3%) decreased from the first to the last inoculation date (from 5/15 to 1/15). In autumn, a year after inoculation, the plants remaining symptomless were monitored for the presence of endophytic *E. amylovora*. Re-isolation on selective medium was successful in 13 plants out of 41. Reisolation was also successful in 7 segments taken from the side shoots developed from the first and second nodes below the inoculation point. The results show that *E. amylovora* can survive a year in symptomless planted scions. Isolation of the mutant from symptomless shoots developed during the growing season following inoculation indicates that *E. amylovora* may have a prolonged symptomless endophytic phase.

Key words: fire blight, latent infection, endophytic survival, *E. amylovora*.

Nursery material, rootstocks and scions may be responsible for short and long distance spread of *Erwinia amylovora* (Van der Zwet, 1994; Van der Zwet and Walter, 1996; Momol *et al.*, 1998). In Italy, *E. amylovora*

was found in association with buds on apple rootstocks (Calzolari *et al.*, 1982), but this material was not an effective source of infection in the orchards where it was planted. A fire blight focus was, on the other hand, found in Emilia Romagna in illegally imported hawthorns (Curto, 1992). No information is as yet available on how symptomless nursery material used for planting in areas free from fire blight may act as a primary source of inoculum, or on the timescale involved. A likely hypothesis is that the bacteria may survive endophytically in symptomless host plants for long periods, causing obvious infections in certain environmental and host plant conditions. Trauma may be a triggering factor (Keil and Van der Zwet, 1972).

The aim of this study was to verify whether the endophytic survival of *E. amylovora* in symptomless pear scions is compatible with their role as a primary source of inoculum in a new orchard.

Bacterial culture. The virulent rifampicin-resistant strain *E. amylovora* Ea 273R1 isolated originally from apple and kindly supplied by Prof. S.V. Beer (Cornell University, Ithaca, New York, USA) was used. The strain was routinely grown at 27°C for 48 h on 5% saccharose nutrient agar (Lelliott and Stead, 1987) with the addition of rifampicin (200 µg ml⁻¹) (Sigma R3501) (selective medium).

Plate count. This was performed on selective medium using tenfold dilutions of a bacterial suspension with A₆₆₀ = 0.060.

Pear scions. Plants of cv. 'Abate Fetel' with a dormant bud grafted on B29 quince root-stock were acquired at the end of winter 1998-1999, transplanted in individual pots and grown in the open on grass. The shoot developing from the bud was trained vertically during the growing season.

Inoculation. The pear plants were placed in a climatic chamber at 25 ± 2°C and the stem was cut transversely 10 cm from the top using pruning shears. Thir-

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ty μ l of bacterial suspension containing 30,000 colony-forming units (cfu) were deposited with a microsyringe (Hamilton 705N) on the fresh wound. Five inoculations were performed at intervals of 10 days from September 21 onwards. On each date, 15 pear scions were inoculated and five pear scions were treated with water as a control. After inoculation, the plants were kept in the climatic chamber for 4 days, then replaced in the open.

Inspections. The plants were inspected weekly until the end of autumn the following year for the presence of basipetal cortical canker starting from the inoculation point, or symptoms of fire blight.

Re-isolation from symptomatic plants. Re-isolation was performed on selective medium and the plates were incubated at 27°C for at least 5 days.

Re-isolation from symptomless plants. Four stem segments 10-cm long were removed with pruning shears from the transverse cut inoculated the previous year. The first segment started from the inoculation point (T_1) and the second (T_2) consisted of the subsequent 10 cm. The third (B_1) and the fourth (B_2) segments were taken respectively from the base of the shoots developed from the two nodes immediately below the inoculation point, B_2 being the lower.

Each segment was washed in tap water and air-dried. Pruning shears were then used to cut the side shoots. After coating the cut surfaces with sterile semi-solid vaseline, the segments were immersed for 10 min in sodium hypochlorite solution (1:4 v/v) and then thoroughly washed in tap water, dried in air and placed under a hood with sterile air. The washing and treatment of the segments with sodium hypochlorite aimed to eliminate contaminating epiphytic bacteria. Coating of the cuts with vaseline aimed to prevent the endophytic bacteria from being killed as a result of hypochlorite diffusion in the tissues. The vaseline-covered ends were then removed and each segment was cut with pruning shears into transverse slices about 3 mm thick. The slices of each segment were placed in sterile 300 ml conical flasks with 50 ml of potassium phosphate buffer (0.01 M; pH 7) (PB), then placed on a rotary shaker (150 rpm) for 45 minutes at 27°C. The washing liquid was collected and centrifuged at 10,000 *g* for 15 minutes. The final pellet was resuspended in 1 ml of PB and used for the re-isolation on selective medium.

Identification. The pink, domed colonies of the mutant grown on the selective medium were selected, puri-

fied on NSA and identified via PCR (McManus and Jones, 1995) and pathogenicity-tested on pear fruitlets kept in a refrigerator at 2°C. The virulent strain of pear *Ea* IPV-BO 1077/7 was used as a positive control.

During the November and December 1999 inspections, 12 symptomatic scions were found with basipetal cankers 1-1.5 cm long starting from the inoculation point (Table 1). The frequency of plants with symptoms varied from 4/15 to 5/15 from the first to the last inoculation date. No control scion with canker was found. All symptomatic scions were destroyed by mid-December.

During the January, February, March and early April inspections, no scion was found with symptoms. On April 12 when growth had resumed and with 5-6 cm long shoots, 4 symptomatic scions were found with characteristic basipetal canker starting from the point of inoculation. No control scion was found with canker or symptoms of fire blight. The total frequencies of symptomatic pears for the five inoculation dates noted by mid-April are given in Table 1. The frequencies decreased from 5/15 inoculations on September 21 and 1 October to 3/15 on October 11 and 1/5 inoculations at the end of October.

During the inspections in the period mid-April to end-October, no symptomatic plants were found with either basipetal canker at the inoculation point or fire blight symptoms. During the same period, no control plant showed symptoms of fire blight.

During October-November, re-isolation of endophytic *E. amylovora* was attempted for all plants inoculated the year before.

Re-isolation was successful from 13 pear trees out of 41 with frequencies varying from 4/5 for the first inoculation date to 1/5 for the last date (Table 2). The frequencies of successful re-isolation from segments of the main stem (T_1 and T_2) and side shoots (B_1 and B_2) varied from 6/10 for the T_1 segments to 3/10 or 4/10 for the B_1 and B_2 segments respectively (Table 4).

The scions used in the experiment were comparable in age and growing practice to those produced in a nursery during a growing season and marketed. The aim of inoculation at the end of the growing season was to produce latently infected scions in which the presence of endophytic *E. amylovora* would be monitored after a year. The brief period in the climatic chamber after inoculation aimed on one hand to ensure establishment of the mutant and on the other to simulate a number of autumn days favourable to late infection.

Basipetal cankers at the inoculation point occurred both during late autumn and at the beginning of the subsequent growing season. Most of the cankers developed by the end of December and their frequency

Table 1. Partial and total frequencies and percentages of symptomatic pears observed during the periods November 1 - December 15 1999 and January 31 - April 15 2000 in relation to inoculation timing.

Inoculation date	Frequency of symptomatic pears		
	A: Nov-Dec	B: Jan-Apr	A+B
Sept. 21	4/15* (26.6%)	1/11 (9%)	5/15 (33%)
Oct. 01	5/15 (33.3%)	0/10 (0%)	5/15 (33%)
Oct. 11	2/15 (13.3%)	1/13 (7.6%)	3/15 (20%)
Oct. 21	1/15 (6.6%)	1/14 (7.1%)	2/15 (13.3%)
Oct. 31	0/15 (0%)	1/15 (6.3%)	1/15 (6.6%)
Total	12/75 (16%)	4/63 (6.3%)	16/75 (21.3%)
Controls	0/25 (0%)	0/25 (0%)	0/25 (0%)

*Number of scions with basipetal cankers/number of inoculated scions.

Table 2. Incidence and percentage of re-isolation of *E. amylovora* mutant from symptomless pear scions a year after inoculation on five specific dates.

Inoculation date	Re-isolation frequency
Sept. 21	4/5*
Oct. 01	3/8
Oct. 11	2/13
Oct. 21	3/10
Oct. 31	1/5
Total	13/41 (31.7%)
Controls	0/10

*Number of scions with basipetal cankers/number of inoculated scions.

Table 3. Frequencies and percentages of re-isolation of the *E. amylovora* mutant from symptomless segments a year after inoculation. T₁= 10 cm segment of the main stem starting from the inoculation cut; T₂= 10 cm segment below T₁; B₁= 10 cm segment at the base of the first side shoot below the inoculation cut; B₂= 10 cm segment at the base of the second side shoot below the inoculation cut.

Stem segment	Re- isolation frequency
T ₁	6/10
T ₂	4/10
B ₁	3/10
B ₂	4/10
Total	17/40 (42.5%)

diminished more or less linearly (data not shown) from the first to the fifth inoculation date. This indicates that late wound-infection during September-October can cause cankers visible before winter, and that their observation becomes more probable the earlier the inocu-

lation date. Few cankers became visible only at the beginning of the growing season and their frequency was similar for the five inoculation dates (9% as against 6-7%). This indicates that for these cankers, factors other than the inoculation date contributed to the infections remaining latent over winter.

The total frequency of pear scions which developed symptoms the following year was 16/75 (21.3%). This indicates that about 1/5 of the scions undergoing wound-infection in late autumn develop basipetal canker from the inoculation point the following year.

Attempts to re-isolate the mutant from symptomless plants a year after autumn inoculation were successful in 13 cases out of 41 (31.7%). This indicates that the mutant was able to survive endophytically in almost a third of the scions inoculated the previous year and remaining symptomless throughout the following growing season. A survival rate of about 30% has also been noted in shoots of recently overwintered symptomless apple trees inoculated the previous year (Crepel *et al.*, 1996). These results show that, following autumn wound inoculation, *E. amylovora* may survive for at least a year within symptomless pear scions. The partial frequency of successful re-isolation from symptomless scions inoculated on September 21 was higher than on other dates (80% as compared with 30-37%). This suggests that survival in symptomless scions is more frequent, the earlier the autumn infection.

The technique of re-isolation by washing does not provide information on the tissue in which survival occurred. The inoculation technique suggests that most bacteria probably penetrated into the wood. It is therefore likely that survival took place in the secondary xylem via the endophytic translocation already known for *E. amylovora* (Van der Zwet, 1994).

The frequency of successful re-isolation of the mutant from T₁ stem segments immediately below the inoculation point was higher than that for other segments. This was to be expected, as those tissues had been directly exposed to contamination. The frequency of successful re-isolation from other segments was similar, in particular from the B₂ lateral segment most distant from the inoculation point.

The re-isolation of *E. amylovora* from symptomless B₁ and B₂ segments grown the following year strongly indicate that the in our symptomless scions *E. amylovora* multiplied and colonised new tissues.

During the study, attempts to re-isolate endophytic bacteria from sections T₁ and T₂ of 17 symptomless plants during the July-August were not successful. In a similar experiment on symptomless apple scions inoculated the previous autumn, re-isolation of the mutant was successful in May and September, but not in July (Ceroni, 1999). These results suggest that *E. amylovora* in symptomless host plants may have an endophytic phase influenced by environmental conditions and/or the physiological state of the host plant (Hickey *et al.*, 1999).

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