

COMPARATIVE STUDY REGARDING *IN VITRO* INFECTIONS WITH *ERWINIA AMYLOVORA* AND *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* ON *POMACEAE* SPECIES

IRINA PARASCHIVA CHIRIAC¹, FL. D. LIPȘA^{1*}, E. ULEA¹

*E-mail: flipsa@uaiasi.ro

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ABSTRACT. The knowledge about pome fruit trees bacterioses and their evolution in orchards is a major objective for plant protection. *Erwinia amylovora* and *Pseudomonas syringae* pv. *syringae* cause on attacked organs of *Pomaceae* species similar dieback symptoms in vegetative and flowering shoots of quince, pear and apple in spring. Both bacteria can produce disastrous diseases in orchards and are therefore of great economic importance. Biological materials represented by vegetative shoots, leaves and fruits of *Pyrus* spp., *Malus* spp., *Cydonia* spp. were used after isolation of different *E. amylovora* and *Ps. syringae* pv. *syringae* strains for *in vitro* infections. Results presented in this study established that for *in vitro* inoculation of *Pomaceae* species similar symptoms in case of leaves and, respectively different symptoms for vegetative shoots and fruits occurred. The occurrence time was the only difference, because *Ps. syringae* pv. *syringae* spread faster than *E. amylovora*. The vegetative shoots inoculated with *E. amylovora*, in comparison to *Ps. syringae*

pv. *syringae*, were more damaged and for both bacteria the highest values for attack degree were recorded in case of pear species, followed by quince and apples, respectively.

Key words: Fire blight; Bacterial blossom blight; Inoculation; Pathogenicity test.

REZUMAT. Studiu comparativ privind infecția *in vitro* cu *Erwinia amylovora* și *Pseudomonas syringae* pv. *syringae* la unele specii din familia *Pomaceae*. Acumularea cunoștințelor despre bacteriozele pomilor semănători și evoluția lor în livezi reprezintă un obiectiv major pentru protecția plantelor. *Erwinia amylovora* și *Pseudomonas syringae* pv. *syringae* produc, pe părțile aeriene ale speciilor din familia *Pomaceae*, simptome caracteristice, care se observă primăvara, în special la nivelul lăstarilor vegetativi și al mugurilor floriferi. Lăstari vegetativi, frunze și fructe de *Pyrus* spp., *Malus* spp. și *Cydonia* spp. au fost utilizate pentru infecții *in vitro* cu tulpini de *Erwinia amylovora* și

¹ Department of Plant Science, Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine, Iași, Romania

Pseudomonas syringae pv. *syringae*. Rezultatele prezentate în acest studiu au stabilit că, pentru inocularea *in vitro* a speciilor de *Pomaceae* cu tulpinile bacteriene, simptomatologia a fost similară în cazul frunzelor și diferită în cazul lăstarilor vegetativi și al fructelor. Timpul scurs până la manifestarea bolii reprezintă o primă diferență între cele două specii bacteriene, în sensul că bacteria *Ps. syringae* pv. *syringae* produce simptome mai repede decât bacteria *E. amylovora*. Lăstarii vegetativi, infectați cu *E. amylovora*, au fost mai afectați, comparativ cu cei infectați cu *Ps. syringae* pv. *syringae* și în cazul ambelor specii bacteriene, cele mai mari valori pentru gradul de atac înregistrându-se la păr, urmat de gutui și măr.

Cuvinte cheie: focul bacterian al rozaceelor; arsura bacteriană comună; inoculare; test de patogenitate.

INTRODUCTION

Erwinia amylovora (Burrill, 1882) Winslow *et al.* (1920) and *Pseudomonas syringae* pv. *syringae* van Hall (1902) are plant pathogenic bacteria commonly associated with diseased pome fruit trees worldwide. *Erwinia amylovora* causes fire blight of many rosaceous plants (Vanneste, 2000), but the most economically important hosts in Romania are apple (*Malus domestica* Borkh.), pear (*Pyrus communis* L.) and quince (*Cydonia oblonga* Mill.). Believed to be native to North America, the Gram-negative bacteria *E. amylovora* is a major quarantine organism currently present in 46 countries around the world (Smith *et al.*, 1997; van der Zwet, 2002). In Romania, fire blight was reported for the first time

in 1992 in two different locations in the south and south east of the country (Severin, 1996). Despite sanitation methods the spreading of the disease could not be stopped and in year 2000 was noticed in all country (Vlad, 2003). *Pseudomonas syringae* pv. *syringae* is an ice nucleation-active bacterium which causes bacterial blossom blight or bacterial blast under appropriate environmental conditions (Renick *et al.*, 2008; Kokoskova *et al.*, 2011).

Studies on *E. amylovora* and *Ps. syringae* pv. *syringae* under natural or controlled infections have aimed to improve disease control based on breeding for resistance (Lespinasse and Aldwinckle, 2000; Luby *et al.*, 2002), determining host susceptibility (Bessho *et al.*, 2001; Bogs *et al.*, 2004), analyzing pathogen strain aggressiveness (Norelli *et al.*, 1988; Cabrefiga and Montesinos, 2005), and evaluating new biological control strategies and products (Johnson and Stockwell, 2000; Cabrefiga *et al.*, 2007).

Both bacteria cause on attacked organs of *Pomaceae* species similar dieback symptoms in vegetative and flowering shoots of pear and apple in spring (Jones and Aldwinckle, 1990). Observable differences are also present, because *Ps. syringae* pv. *syringae* produced infection more frequently in blossoms, and these rarely progress to stalk flowers and skeleton branches.

The aim of this research was to establish which bacteria produces the first symptoms on shoots, leaves and

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fruits of *Pomaceae* species with purpose to enhance the professionally diagnose of the pome fruit pathogens under Romanian climatic conditions.

MATERIAL AND METHODS

Samples to isolate and identify bacteria were harvested from pomological collection of "Vasile Adamachi" farm, which belongs to University of Agricultural Science and Veterinary Medicine Iași (Romania) and from a nursery stock located also in Iași. Herbaceous shoots were collected from apple, pear and quince species. Sampling was done in early June and the samples processing took place in the phytopathological laboratory.

From the samples with typical fire blight symptoms, fragments of 1 - 2 cm were taken in case of shoots and leaves. Fragments between the necrotic and healthy tissue from leaves were used. Samples were chopped in sterile Petri dishes and mixed with 1mL distilled water. The mixture was allowed 15 minutes to rest, so that the bacteria could spread. In the next step, mixture was poured on two different growth nutrient media (NSA - nutrient sucrose agar and King B) and on pear slices (King *et al.*, 1954; San *et al.*, 2009).

To confirm the presence of *Erwinia amylovora* pathogenicity test was performed. Pathogenicity tests were carried out on immature pear fruit, as described by Beer and Rundle (1983). Immature pear fruits were collected during June; surface sterilized with 70% ethanol and cut into transverse slices 0.5 cm thick. For each strain, three slices, each from a different fruit, were placed on a sterile moist filter paper, in a sterile plastic dish. 30 μ l of bacterial suspension,

at a concentration of 2×10^8 cells/ml, was added. Bacterial inoculums were prepared by growing each strain overnight on nutrient agar plate, followed by dilution with 0.05 M buffer phosphate (pH 6.5). The slices were maintained under humid conditions at 25°C. Production of ooze on the surface of the slice was observed during 5 days.

In order to perform *in vitro* infections, herbaceous shoots (25-30 cm length), leaves and fruits of apple, pear and quince from trees without fire blight symptoms were harvested. Inoculums with a dilution of 10^{-1} from both bacteria for the *in vitro* infections were prepared. Shoots infections were carried out by injecting 0.1 mL dilution in the apical bud and four stings in upper leaves. Shoots leaves were injured with a sterile needle dipped in dilution (Severin and Cornea, 2009). Inoculation of detached leaves was done similar to shoots leaves. Fruits were inoculated using also a sterile needle dipped in dilution and for each fruit were made five stings.

In the experimental room, conditions for maintaining the relative humidity close to 90% and the temperature in range from 28° to 32°C were created. Visual observations were made to record all changes after *in vitro* infections.

RESULTS AND DISCUSSION

After observing all the Petri dishes in ultraviolet light, fluorescent colonies were found only on pear samples and on King B medium. NSA and King B culture media are used to diagnose the bacterium *Erwinia amylovora*, but they also highlight *Pseudomonas syringae* pv. *syringae* due to the substances which they

contain. King B allows the production of fluorescein (or pyoverdin), a yellow - green pigment that fluoresces under ultraviolet light. In certain strains of *Pseudomonas* spp., dipotassium phosphate increases the concentration of phosphorus which stimulates the production of fluorescein and inhibits the pyocyanin production. On this culture media, fluorescent colonies could be seen by naked eye, in comparison to NSA media where fluorescent colonies can be seen only in ultraviolet light. On King B nutritive media, *Erwinia amylovora* develop white circular colonies, type S (smooth), with sharp edges, while on NSA media the circular colonies are yellow white,

type S and slightly convex (Paulin and Samson, 1973; Billing, 1974).

After 72 hours, only on three from the four analyzed samples pear slices appeared numerous white-yellowish colonies with milky consistence. The colonies presence on pear slices surface (Fig. 1) confirm the existence of *Erwinia amylovora* bacteria in the processed samples of *Pomaceae* species. *E. amylovora* was isolated from quince (E.A. - A.G.) and pear (E.A.- A.P.) samples harvested from pomological collection, and from quince sample from nursery stock (E.A. - S.G.). *Pseudomonas syringae* pv. *syringae* was isolated only from pear samples. None of the two bacteria was isolated from apple samples.

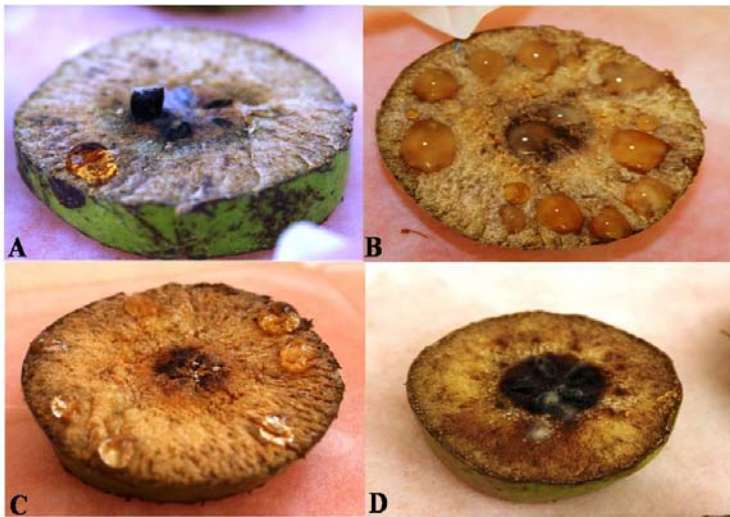


Figure 1: A - *Erwinia amylovora* ooze drop on pear slice from quince sample harvested from pomological collection; B - *Erwinia amylovora* ooze drops on pear slice from quince sample harvested from nursery stock; C - *Erwinia amylovora* ooze drops on pear slice from pear samples harvested from pomological collection; D - No ooze drops on pear slice surface from apple samples harvested from pomological collection.

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Drops of bacterial ooze were reported on pear and quince shoots inoculated with different *E. amylovora* strains. Pear shoots showed droplets on stem, buds and petiole. Quince shoots inoculated with E.A. - A.G. strain showed ooze drops on all organs (less on the buds), while quince shoots inoculated with E.A. -

S.G. showed ooze drops only on stipules. No bacterial droplets were present on apple shoots. The time range in which bacterial exudates occurs on shoots infected *in vitro* with different strains of *E. amylovora* were identified (Table 1). Ooze droplets appeared yellow on stem and stipules and orange on buds and petiole.

Table 1 - Occurrence of bacterial exudate on shoots *in vitro* infected by different strains of *Erwinia amylovora*

Species/shoots infected <i>in vitro</i>	Strains used for <i>in vitro</i> inoculation	Time range in which bacterial droplets were identified (hours)				
		12	48	72	96	108
Quince shoots	E.A. - S.G.	-	-	++	-	-
Apple shoots	E.A. - S.G.	-	-	-	-	-
Quince shoots	E.A. - A.G.	+	++	-	-	-
Pear shoots	E.A. - A.P.	+	+	+	+	++

Abbreviation: + – one bacterial colony; ++ – two bacterial colonies; - – colony absent; E.A. - A.G. – *Erwinia amylovora* isolated from quince samples; E.A. - A.P. – *Erwinia amylovora* isolated from pear samples; E.A. - S.G. – *Erwinia amylovora* isolated from nursery stock

Detached organs are the most suitable plant material to inoculate quarantine-level pathogens, because they allow biological material manipulation under accurate and safe conditions (Abdollahi *et al.*, 2004; Denman *et al.*, 2005).

Pear and quince shoots inoculated with different *Erwinia amylovora* strains, showed drops of bacterial ooze on all biological parts. During favourable conditions, ooze drops containing numerous bacteria appear on stalks (Ulea, 2003). These symptoms could be observed in both cases, on *in vivo* and *in vitro* infection. Systemic infected shoots could be identified in the early stages of attack because of buds orange pigmentation (van der Zwet and Beer,

1995). Depending on climatic conditions, the bacterial ooze may take different forms, but the most common is the liquid form (van der Zwet and Beer, 2002). The liquid form of bacterial exudates was reported in controlled environmental conditions on stem, stipules, petioles and buds inoculated with different strains of *Erwinia amylovora* (Fig. 2). Inside the pomes fruit trees, *E. amylovora* advances fast to stem that looks at the beginning watery and later the colour is changed in dark brown. The shoots present leaf and stipules necrosis. "Shepherd's crook" or "crutch" symptom was observed only on quince shoots inoculated with E.A. - A.G. and E.A. - S.G. (Fig. 3).

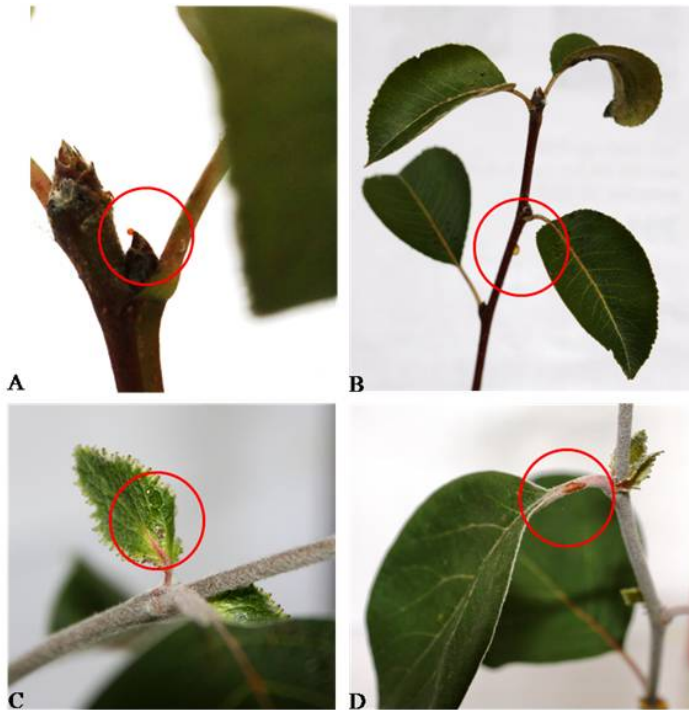


Figure 2: A - orange coloured ooze drop of *E. amylovora* on bud, B - yellow coloured ooze drop of *E. amylovora* on stem, C - yellow coloured ooze drop of *E. amylovora* on stipules, D - orange coloured ooze drop of *E. amylovora* on petiole



Figure 3 - "Shepherd's crook" symptom produced on quince shoots: A - six day after inoculation; B - seven day after inoculation; C - eight day after inoculation; D - nine day after inoculation; E - ten day after inoculation

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The apple, pear and quince vegetative shoots inoculated with *Ps. syringae* pv. *syringae*, showed no ooze drops and no symptom of “crutches”. The only changes observed were necrosis on leaves and stipules.

Detached leaves inoculated with *E. amylovora* and *Ps. syringae* pv. *syringae* showed similar symptoms, which appears very fast. Inoculated leaves with *E. amylovora* showed symptoms beginning with the third day after inoculation and after two days more were almost completely necrotic. From the fourth day after inoculation with *Ps. syringae* pv. *syringae* the apple leaves were fully necrotic. A similar situation was observed on pear leaves infected with E.A. - A.P. and *Ps. syringae* pv. *syringae*. Burning symptoms spread rapidly and no other changes were reported. In case of quince leaves infected with both bacteria, the results showed that *Ps. syringae* pv. *syringae* produced necrosis symptoms much faster than *E. amylovora*. *In vitro* inoculated leaves proved to be the most susceptible organs of the pome fruits trees infected with both bacteria.

Ps. syringae pv. *syringae* did not produce significant damages on *Pomaceae* fruit inoculated *in vitro*. Quince and apples inoculated with E.A. - S.G. and E.A. - A.G. presented necrosis with different shapes and sizes on the area next to the needle. Diameter of necrosis in all *in vitro* inoculated fruits showed values between 0.5 mm and 1.0 mm.

Differences were observed between pear fruits inoculated with

E.A. - A.P. strain and *Pseudomonas syringae* pv. *syringae*. Since pathogenicity test is performed on immature pear fruit and all the fruits used in the experiment were immature, is justified why symptoms on inoculated pear fruits with E.A. - A.P. are more obvious than on the other species.

The greatest damage caused by *E. amylovora* was recorded at leaves level, followed by vegetative shoots and fruits. The assessment of attack degree on shoots produced *in vitro* condition provided values from 8.6 to 74.6% in case of apple and pear species. Apple, pear and quince leaves infected with *E. amylovora* showed a significant attack degree, the percentage for pear species reaching up to 93.3% (Fig. 4).

After assessing the results on vegetative shoots inoculated *in vitro* with *Ps. syringae* pv. *syringae* the conclusion was that the *Pomaceae* species responded differently. Apple shoots proved to be the most resistant and pear shoots the most susceptible. This bacterium produced on apple and pear leaves a 100% damages. Artificially inoculated fruits presented no significant changes during the experiment (Fig. 5).

Azegami *et al.* (2006) mentioned that *E. amylovora* multiply better in succulent shoots in early summer than in fruit-bearing twigs in late summer, when apples are ready to mature. In case of infected fruits bacteria can cross fruits epicarp, reaches and spreads within them during their maturation, although there are no visible symptoms to the surface.

Schroth *et al.* (1974) believes that infected fruits are important in the life cycle of *Erwinia amylovora*. It was not scientifically demonstrated that mature fruits infected, play an important role in long-distance transmission of bacteria, although McLarty (1923), Anderson (1952) and

Goodman (1954) suggested that this is possible.

From all detached organs, the most sensitive were the leaves inoculated with *Pseudomonas syringae* pv. *syringae* and the pear shoots inoculated with *Erwinia amylovora*.

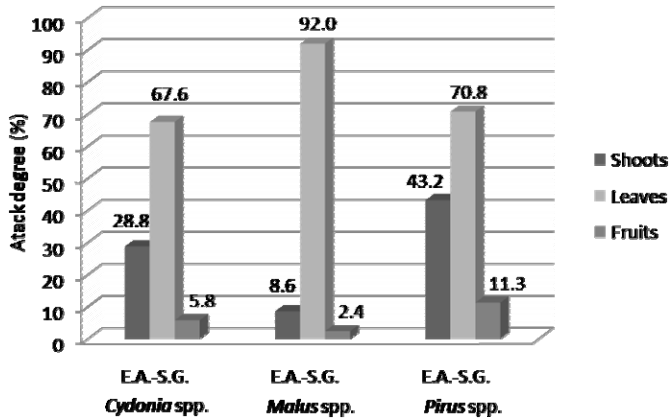


Figure 4 - Attack degree of the pathogen *Erwinia amylovora* on *Pomaceae* species

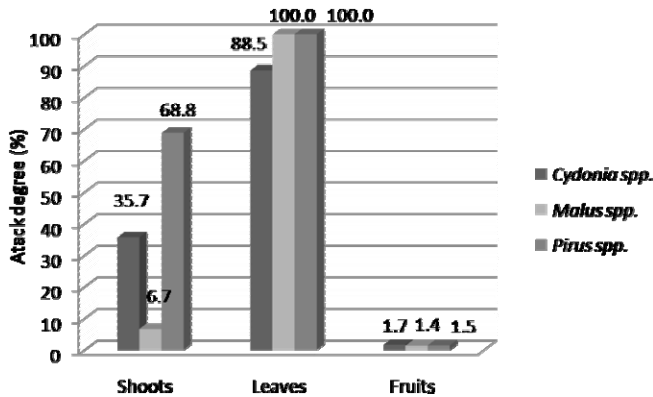


Figure 5 - Attack degree of the pathogen *Pseudomonas syringae* pv. *syringae* on *Pomaceae* species

CONCLUSIONS

E. amylovora and *Ps. syringae* pv. *syringae* showed similar symptoms on *in vitro* inoculated

leaves. The occurrence time was the only difference, because *Ps. syringae* pv. *syringae* spread faster than *E. amylovora*. The vegetative shoots inoculated with *E. amylovora*, in

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comparison to *Ps. syringae* pv. *syringae* were more damaged and for both bacteria the highest values for attack degree were recorded in case of pear species, followed by quince and apples, respectively.

The form of crutch has been reported only on quince shoots inoculated with different strains of *Erwinia amylovora*. Typical symptoms of fire blight were observed predominantly on pear and quince vegetative shoots, and less in case of apple trees. Due to favorable *in vitro* conditions bacteria *E. amylovora* was able to multiply rapidly and caused appearance on quince and pear vegetative shoots of bacterial exudates. In case of fruits inoculation, bacteria showed typical symptoms only on immature pear.

The apple, pear and quince trees shoots inoculated with *Ps. syringae* pv. *syringae* presented no ooze drops or any form of crutch. The apple and pear leaves inoculated *in vitro* presented necrosis on all leaf area.

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