Testing the virulence of some Hungarian *Erwinia amylovora* strains on *in vitro* cultured apple rootstocks

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Hevesi M.[1], Papp J.[2], Jámbor-Benczúr E.[3], Kaszáné Csizmár K.[1], Pozsgai I.[3], Gazdag Gy.[3] and Balla I.[4]

Szent István University, Faculty of Horticultural Science
[1]Department of Plant Pathology
[2]Department of Pomology
[3]Department of Floriculture and Dendrology
[4]Research Institute for Fruitgrowing and Ornamental Plants
H-1518 Budapest, P. O. box 53.

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Summary: A useful method was improved to test and to evaluate the susceptibility of plants to fire blight and the virulence of *E. amylovora* strains. Six Hungarian strains from different host plants were tested on *in vitro* cultured apple rootstocks. Disease rating was used for the characterization of the process of disease development. The different strains had different capacity to cause disease, mainly in the first period of incubation. There were significant differences between the virulence of the strains.

Introduction

The fire blight is a very important and dangerous disease which has been spreading from North-America and appeared in Europe in the middle of this century. It causes deadly damages on the woody plants of *Rosaceae* family, *Pomoideae* subfamily *(Van der Zwet & Keil,* 1979).

The bacterium was originally detected in apple orchard in Hungary *(Hevesi,* 1996). Since the earliest observation of fire blight the pathogen had been isolated from various susceptible plant species like fruit trees and ornamental plants *(Hevesi & El-Arabi,* 1998).

For this reason it was decided to test the virulence of the strains isolated in Hungary from different host plants. Because of the strict quarantine orders it was advisable to make the plant inoculations *in vitro*. In the literature, some different *in vitro* testing methods are described.

Geider et al. (1991) tested some strains of *E. amylovora* on agar embedded *in vitro* pear cells. *Paulin & Duron* (1986) used root calluses of *Malus domestica* cultivar *Golden Delicious* for identification of pathogenic strains of *E. amylowora*. They worked out a method for inoculation the calluses with the pathogen.

Duron & Paulin (1987) described a quick testing method on *in vitro* cultured shoots of pear cultivars to compare the susceptibility of them. The duration of their experiment was up to 2 weeks. They used a special necrosis severity scale and necrosis severity index (NSI) to describe the process of disease and observed the plants in 2–3 days. They stated that this method was useful for quick testing and showed the difference between the susceptible and resistant plants *in vitro*.

Viseur & Tapia (1987) described a similar *in vitro* testing method for apple and pear. They tested different artificial inoculation methods and concluded that the more uniform and reproducible symptoms were obtained by cutting the leaf apex with scissors dipped in the bacterium suspension. They used a different scale for the observation of the symptoms and stated that the *in vitro* method proved to be useful to establish a clear distinction between highly resistant and very susceptible cultivars.

Norelli et al. (1987) also described a method on rooted *in vitro* plantlets of apple cultivar *Novole*. They tested a lot of different *E. amylovora* strains in greenhouse as well and found significant association between data gained *in vitro* and in greenhouse, mainly in the first period of the disease. The finally, the growth of the bacteria was depend on the different conditions in greenhouse or *in vitro*.

The *in vitro* testing method is used successfully nowadays as well to test the fire blight resistance among transgenic pear clones *(Reynoird* et al., 1999).

About susceptibility of the apple rootstocks several different data can be found. According to *Huet & Michelesi* (1990), the M.26 rootstock proved to be susceptible and MM.106 was even more susceptible. *Fisher* (1996) & *Hrotkó* (1997) stated that the M.26 rootstock had no resistance against the bacterium and the MM.106 had a weak resistance.

It is important, that the strains isolated from hawthorn (*Crataegus*) caused disease on pear and apple *(Berris,* 1995). The cross contamination is also possible in the case of strains isolated from ornamental plants as well.

The susceptibility of the hosts was described mostly from plants that were grown in orchards and contaminated by natural way. The results are influenced by the soil type, weather, microclimatic conditions, cultural practices, pest and disease control, tree age and physiological condition of the trees, etc. The *in vitro* testing method eliminates the mentioned environmental effects. The tested plants are vigorous and had the same age and they are uniform from the point of view of physiological condition.

The aim of our research was to work out a quick, safe and reliable method for testing the *E. amylovora* on *in vitro* cultured plants with describing the process of the development of the disease. We wanted to compare the susceptibility of the rootstocks and to evaluate the virulence of the different bacterial strains isolated in Hungary.

Material and method

In vitro cultured shoots of M.26 and MM.106 apple rootstocks were used as test plants. The *in vitro* plants originated from the Research Institute of Fruit Growing and Ornamental Plants (GYDKFV, Budapest). The shoots were propagated before inoculation on A1 medium which consisted of MS macroelements in half concentration, MS microelements and vitamins in full concentration *(Murashige & Skoog,* 1962) BA 0.25 mg^{-1}, IBA 0.1 mg^{-1}, sucrose 20 g^{-1}, Difco-bacto agar 7 g^{-1}. The pH was adjusted with KOH to 5.6.

The plantlets were passed on the same medium before inoculation. Two plantlets of 20–25 mm height were placed in every Erlenmeyer flask which contained 30 ml of the medium and was covered by three layers of 0.017 mm plastic foil.

The cultures were illuminated by white light of 40 $\mu M/m^2/s$ using 16/8 hour light/dark cycles. The temperature was 24–26 °C and 20–22 °C during the light and dark periods respectively. The inoculation was made on the 7^{th} day after passing the plantlets onto fresh medium.

During the experiment six strains of *E. amylovora* were tested on the rootstocks *(Table 1).* The bacterial suspension contained 10^8 ml^{-1} cells. The inoculation was made by a scissor immersed in the suspension and the apex of the leaves of the plantlets was cut down in reversed position.

The origin of the strains and the tested rootstocks are introduced in *Table 1.*

Table 1 The origin of the *Erwinia amylovora* strains used and the tested rootstocks

E. amylovora strain	Host plant	Rootstock
Ea1	*Malus domestica*	M.26, MM.106
Ea10	*Pyrus domestica*	M.26, MM.106
Ea17	*Cydonia oblonga*	M.26
Ea28	*Crataegus monogyna*	M.26
Ea30	*Cotoneaster sp.*	M.26
Ea31	*Pyracantha sp.*	M.26

Every treatment contained 10 plants and was repeated 3 times. The duration of the experiments was four weeks and the plantlets were observed weekly.

For evaluation of the data disease percent and disease rating were used.

For the estimation of the disease severity a special disease rating (DR) system was worked out. The diseased plants were divided into four categories based on the severity of the disease presented. The system is a modification of the Horsfall-Barratt concept *(Horsfall & Barrat,* 1945).

The disease categories were determined as follows:

1. Browning of the leaf tissues near the cut part and the vein.
2. Browning of 2–3 other leaves near the cut leaf or the apex of the shoot.
3. Browning of half part of the shoot
4. Browning of the whole stem and appearing bacterial exudate on the shoot and near the surface of the medium *(Fig. 1).*

The data were evaluated by calculation of DR, described by *Bertrand & Gottwald* (1978). They used 10 categories for describing the state of the diseased leaves caused by fungus. With modification of this method a special DR was worked out:

$$\text{Disease rating} = \frac{\Sigma\,[(N1 \times 1)+(N2 \times 2)+(N3 \times 3)+(N4 \times 4)]}{\Sigma\ N}$$

N1–4: Number of plants in each disease category.

The calculation of the disease rating was fundamental because this method followed the disease process by a more exact way, compared to the disease percentage.

Results

The inoculation of *in vitro* plants with *E. amylovora* strains was successful because it was possible to evaluate the virulence of the strains and the susceptibility of the plants.

According to the data, both rotstocks were susceptible but not in the same level *(Table 2* and *Figs. 2–3).* The MM.106 rootstock had a higher susceptibility than the M.26.

There was a slight difference in the virulence between Ea1 and Ea10 strains. The Ea10 strain (isolated from pear) caused weak but finally strong disease symptoms compared to the Ea1 strain (isolated from apple) during the first half period of the experiment.

Figure 1 The symptoms caused by different *Erwinia amylovora* strains on *in vitro* cultured shoots of M.26 apple rootstock four weeks after the inoculation. A. Disease category 2. (on the left plant) and 4. (on the right plant), caused by Ea28 strain. B. Disease category 3 (on the left plant) and a healthy plant (on the right), inoculated by Ea30 strain. C. Disease category 4 (on the left plant) and a healthy plant (on the right) inoculated by Ea31 strain.

Table 2 The disease percent of M.26 and MM.106 apple rootstocks inoculated by *E. amylovora* strains

		Disease % Weeks after inoculation			
strains	rootstocks	1.	2.	3.	4.
Ea1	M.26	58	68	68	68
Ea1	MM.106	65	78	78	78
Ea10	M.26	40	50	65	80
Ea10	MM.106	39	50	67	89

Table 3 The disease percent of M.26 rootstock inoculated by *E. amylovora* strains

	Disease % Weeks after inoculation			
strains	1.	2.	3.	4.
Ea17	38	75	88	88
Ea28	13	81	87	93
Ea30	31	94	94	94
Ea 31	50	75	81	94

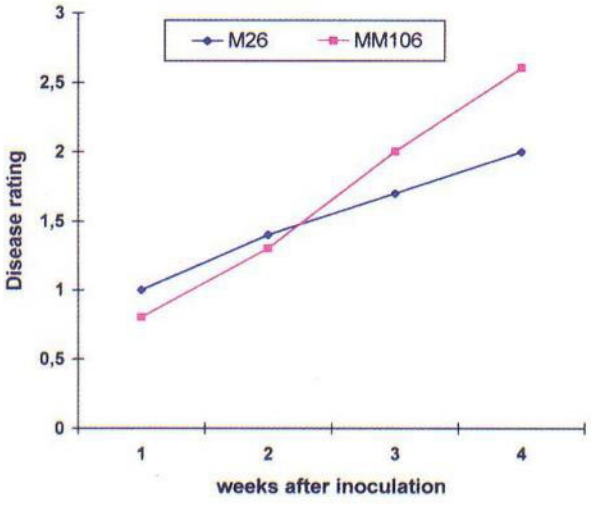

Figure 2 The disease development is shown by disease rating on M.26 and MM.106 apple rootstock inoculated with Ea1 (apple) strain of *Erwinia amylovora*.

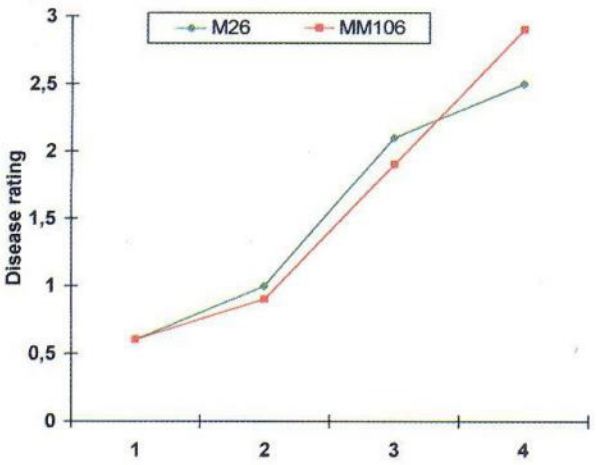

Figure 3 The disease development is shown by disease rating on M.26 and MM.106 apple rootstock inoculated with Ea10 (pear) strain of *Erwinia amylovora*.

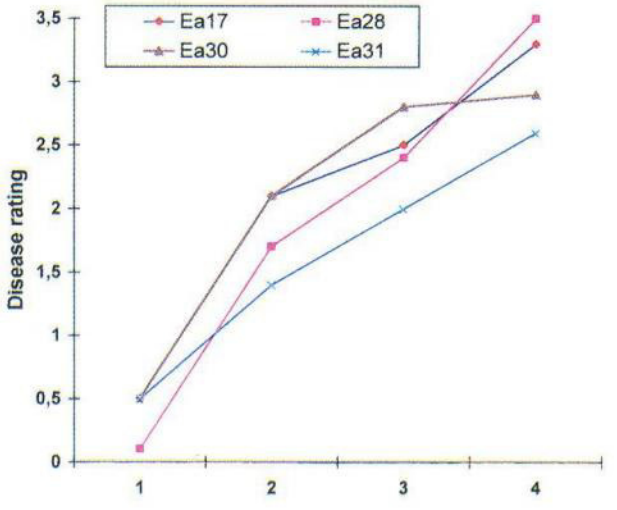

Figure 4 The disease development is shown by disease rating on M.26 rootstock inoculated with Ea17 *(Cydonia)*, Ea28 *(Crataegus)*, Ea30 (Cotoneaster) and Ea31 (Pyracantha) strains of *Erwinia amylovora*.

As only a little difference was found in the susceptibility of the two rootstocks the next experiment was carried out with M.26 rootstock. The rootstock proved to be susceptible to the further *E. amylovora* strains as well. In the case of other *E. amylovora* strains isolated from different ornamental plants, similar tendency of virulence was observed as in the case of Ea10 strain. The disease rating showed the same tendency as the contamination percentage, but a more sensitive way. *(Table 3, Figs. 1, 4).*

It was surprising that in the first period of observation the Ea28 strain (from *Crataegus*) seemed to be less virulent but at the end this strain proved to be the most virulent. The Ea31 strain (from *Pyracantha*) seemed to be the least virulent of all.

Conclusions

We improved a useful method for testing the virulence and describing the disease process of *E. amylovora* on *in vitro* cultured shoots of plantlets.

The advantages of the *in vitro* method are:

1. Safe, because the whole testing can make *in vitro*.
2. Quick and cheap, because the duration is only a month and 20-25 mm high *in vitro* plants were used.
3. The culture environment is standard in contrast to the field experiments.
4. The *in vitro* plants are in a juvenile stage so that the physiological state of the test plants is the same. It is important because the main differences of the field experiments concerning the susceptibility of the same cultivar are influenced by the different physiological states of the plants.

We developed special disease categories based on the severity of infection presented for estimating the disease severity of the inoculated plantlets. We observed the plantlets for a longer period compared to the other authors *(Duron & Paulin,* 1987, *Norelli* et. al, 1987, *Viseur & Tapia,* 1987). We followed the complete development of the disease. We found differences not only in the first but in the final period of the disease as well.

Concerning the susceptibility of M.26 and MM.106 apple rootstocks we came to conclusions similar to that of *Huet & Michelesi* (1990).

It was interesting that the bacterial strains isolated from different host plants showed different capability in virulence not only in the first period of incubation. There were significant differences in virulence between the strains isolated from apple and from the other host plants.

Our results justified that the bacterium strain isolated from *Crataegus* (according to *Berris,* 1995) was able to cause serious disease on apple rootstocks and it seemed to be very agressive, especially in the final period of the experiments. In addition to the strains originating from *Pyracantha* and *Cotoneaster* proved to be virulent, too.

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