



Theses of PhD dissertation

**SOFT ROT AND FIRE BLIGHT SUSCEPTIBILITY OF
IN VITRO POTATO AND APPLE PLANTLETS**

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1. INTRODUCTION

Among biotic stress factors, the bacterial diseases including *Erwinia* species caused significant yield losses in Hungary in the last years.

Symptoms caused by different *Erwinia* species are completely different. *Pectobacterium carotovorum* subsp. *carotovorum* (syn. *Erwinia carotovora* subsp. *carotovora*) and *Dickeya dadantii* (syn. *Erwinia chrysanthemi*) are the bacterium species, which attack potato causing so called „soft-rot”. In contrast, *Erwinia amylovora* causes fire blight on infected apple.

They are widespread in the world and can cause significant economic losses worldwide. There is no effective protection method against to them yet. Involvement of resistant cultivars into agricultural production would be a solution.

Several contradicting results have been reported in the literature about biochemical changes induced by infection in *in vitro* conditions. Therefore we considered to be important task to study the host-responses of plants to biotic stress including biochemical markers. Our aim was to clarify that responses of plants to different diseases are the same or different. These investigations required special conditions because biochemical changes can be tracked only on plants, which grow under the same conditions, such as *in vitro* plantlets.

2. THE AIMS OF RESEARCH WORK

Implementation of resistance-tests and biochemical analysis, and further development of test-methods were the main objects of our PhD research work.

The following specific tasks were intended to solve:

1. Studying of susceptibility/resistance traits (degree of biotic stress tolerance) of potato cultivars/clones and apple cultivars on micropropagated plants.
2. Selection of resistant, moderate susceptible and very susceptible cultivars/clones for biochemical model-experiments.
3. Tracking and comparing of biochemical process accompanying different bacterial symptoms (soft-rot, fire blight).
4. Revealing of congenialities or differences in plant responses to infection (defense mechanism) using biochemical markers.
5. Studying of suitability of micropropagated plantlets for susceptibility/resistance tests.
6. Working out rapid and reliable test methods applicable on micropropagated plantlets.

3. MATERIAL AND METHODS

3.1. Bacteria

Suspensions of virulent strains of the *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*), *Dickeya dadantii* (*Dd*) and *Erwinia amylovora* (*Ea*) were used in our experiments in concentration of 10^8 cells ml^{-1} .

3.2. Plant material

Plant material were micropropagated for the experiments in the Biotechnology Laboratory of Research Institute of Nyíregyháza belonging to the Debrecen University. Potato genotypes involved in experiments were following: 77365/103, 98/91, 136/92, 36/92, 34/85, 736/82, 1469/83, 77399/514, ‘Desiree’, ‘Réka’, ‘Cleopátra’, ‘Rachel’ és a ‘Boró’). Apple cultivars (‘Red Fuji’, ‘Freedom’, ‘Húsvéti rozmaring’, ‘Jonagold’, ‘Hesztia’, ‘Idared’ és ‘Tenroy’ (Royal Gala)) were also studied.

3.3. Test methods for infection and evaluation of symptoms

We developed a new method for *in vitro* infection of potato (shoot and tuber). *In vivo* potato infections were made by use of method reported earlier by Vlasov and Pereverzev (1989). Infection of *in vitro* apple shoots were made by method described by Hevesi *et al.* (2000).

Three-week-old potato plantlets were used for ***in vitro* shoot infection of potato cultivars**. Symptoms on leaves and stems were

observed at the 7th day after infection, and index of infection (F_i) was calculated from results according to the formula:

$$F_i = \frac{\Sigma [(N_1 \times 1) + (N_2 \times 2) + (N_3 \times 3) + (N_4 \times 4) + (N_5 \times 5)]}{\Sigma N}$$

where: N_{1-5} : number of diseased plants belonging to the given scale-degree .

ΣN : the number of total observed plants

According to the F_i value potato cultivars were classified as resistant (F_i), or moderately resistant, or moderately susceptible, or susceptible or very susceptible against to the given bacterium.

Samples for **counting of bacterium cells** were collected from the site of inoculation (SZ), from the part of stem above the inoculation (F) and from the part of stem below the inoculation (A). 1-1 cm long pieces of stem were excised from sample sites and three pieces of stem per genotype were used for initiation of bacterium culture. Bacterium cultures were incubated at 26 °C for 48 h and then colonies developed were counted.

Infection of potato microtuber was made by sterile injection needle dipped into bacterium suspension: tubers were pierced and then placed onto wet filter paper in Petri dish. Evaluation were made at the first, at the third and at the seventh days after infection.

Discs of potato tuber were used for **infection of primer tubers**, and the weight of discs was measured previously. After infection the discs were placed into Petri dishes and incubated at 26 °C for 24-26 h.

The next day, rotted plant tissue was removed by washing and the weight of remaining (healthy) plant tissue was measured. Differences between the weight of potato discs after and before of inoculation was the base for determination of the degree of susceptibility. Results were expressed as the rate of healthy tissue weight (%).

Inoculation of micropropagated apple shoots was made by scissors dipped into bacterium suspension; the second, fully developed leaves from the shoot tips were cutted in half. Susceptibility of *in vitro* apple shoots were evaluated at the second, at the fifth and at the eighth days after inoculation. The degree of the disease was determined on the browning rates of tissues on the cutted leaves, and on the other leaves, and shoots infected systemically.

3.4. Biochemical analysis

Genotypes for biochemical analysis were selected according to the results of *in vitro* plantlets infection as follows: in the case of *Pectobacterium carotovorum* subsp. *carotovorum*: 77365/103 (resistant), 36/92 (moderately susceptible), 98/91 (very susceptible). In the case of *Dickeya dadantii*: 34/85 (resistant), 'Réka' (moderately susceptible), 'Boró' (very susceptible). In the case of *Erwinia*

amylovora: ‘Freedom’ (resistant), ‘Húsvéti rozmaring’ (moderately susceptible), ‘Tenroy’ (very susceptible).

Determination of peroxidase activity was performed in the Corvinus University of Budapest, Department of Applied Chemistry. The POD activity was measured by spectrophotometrical method in the presence of H₂O₂, as substrate and ortho-dianisidine as chromogenic reagent ($\epsilon=11,3$) (Shannon *et al.*, 1966). The increase of absorbance was measured at 460 nm. The enzyme activity was calculated on fresh weight and it was given in U/mg.

Determination of carbohydrate content was realized in the HPLC laboratory of the Department of Fruit Science belonging also to the Corvinus University of Budapest.

Statistical analysis of results were made by one-way ANOVA using SPSS 13.0 for Windows software. Homogenous groups were formed by Tukey-test.

4. RESULTS AND DISCUSSION

4.1. Susceptibility of potato genotypes

Based on the results observed at 7th day after infection of *in vitro* potato shoots we can conclude that genotypes can be classified into four or five groups considering their susceptibility for *Pcc* or *Dd*, respectively.

The majority of genotypes proved to be moderately resistant or moderately susceptible (Table 1).

Table 1. Susceptibility of *in vitro* potato shoots to *Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya dadantii*.

Classification	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	<i>Dickeya dadantii</i>
Resistant	77365/103, 'Rachel'	34/85
Moderately resistant	77399/514, 'Réka', 'Cleopátra'	1469/83, 77399/514, 'Cleopátra', 'Rachel'
Moderately susceptible	'Desiree', 1469/83, 36/92	77365/103, 36/92, 98/91, 736/82, 'Desiree', 'Réka'
Susceptible	136/92, 736/82	
Very susceptible	'Boró', 34/85, 98/91	'Boró', 136/92

In short, 'Rachel' and 77365/103 proved to be resistant, while 'Boró', 34/85 and 98/91 proved to be the most susceptible to *Pcc*. Only 34/85 clones showed resistance against to *Dd*, while 'Boró' and the 136/92 were very susceptible.

Comparing results obtained in experiments including *in vitro* potato plantlets, we can conclude that *Pcc* was more aggressive and resulted in more severe symptoms than *Dd*. Some genotypes responded similarly to both bacteria as follows: 77399/514 and the 'Cleopátra' were

moderately resistant, 36/92 and the ‘Desiree’ were moderately susceptible, while ‘Boró’ was very susceptible for both bacteria species. Bacterial cell number of *Pcc* was determined on bacterium cultures isolated back from infected plant tissue at the 3rd and 7th day after infection, concomitantly with observations.

In the case of isolation made at the 3rd day the most bacteria colonies grown when isolation was made from the site of infection (SZ), and it was true for each tested genotype (Figure 1). Comparing potato genotypes the most bacterial colonies were observed on isolates originated from 98/91 including each sample site, and this result is accordance with results of shoot infection.

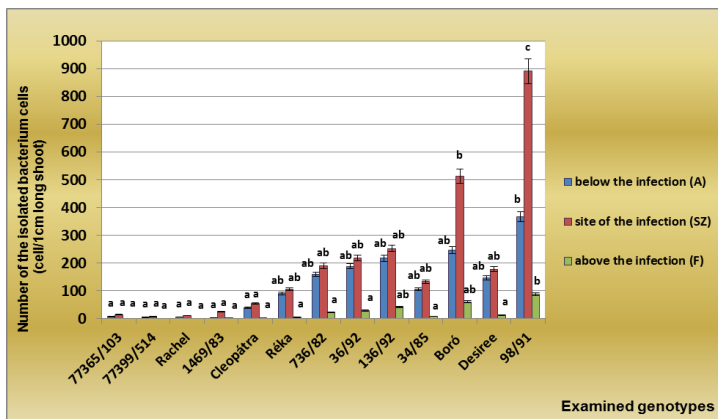


Figure 1. The number of cells of *Pectobacterium carotovorum* subsp. *carotovorum* isolated at the 3rd day after infection from *in vitro* potato shoots.

In contrast, when isolation was made at the 7th day after infection, the most bacterial colonies grown on cultures isolated from the sites below infection (A) (Figure 2).

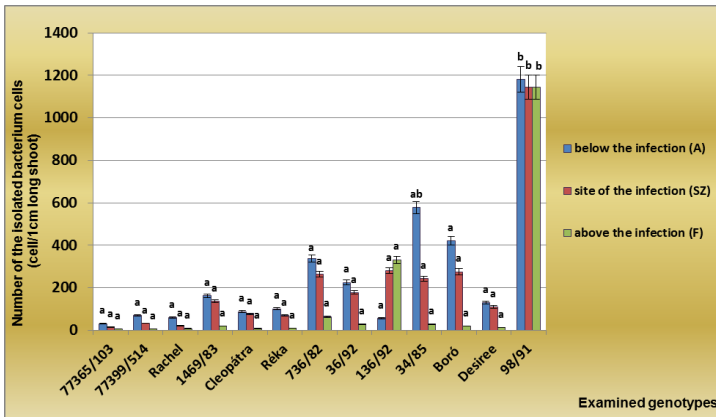


Figure 2. The number of cells of *Pectobacterium carotovorum* subsp. *carotovorum* isolated at the 7th day after infection from *in vitro* potato shoots.

In the case of *Dd* the most bacteria colonies grown when isolation was made from the site of infection (SZ) either at the 3rd or 7th day. However, similarly to *Pcc*, the number of bacterial cells isolated from the below infection (A) at the 7th day also showed an increase. These results suggest that infection spread in the stem downwards. Moreover, it seemed that multiplication and spreading of *Dd* in shoot is slower than that of *Pcc*. It can be supposed that it is the reason for the phenomenon that symptoms of *Dd* on *in vitro* potato shoots were less severe than symptoms of *Pcc*.

Grouping of genotypes were based on the symptoms observed at the 7th day after infection of microtubers as listed in the Table 2.

Table 2. Susceptibility of *in vitro* potato tubers to *Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya dadantii*.

Classification	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	<i>Dickeya dadantii</i>
Resistant	‘Rachel’	77399/514
Moderately resistant		136/92, 34/85, 1469/83, 36/92, 736/82, ‘Réka’
Moderately susceptible	34/85, 1469/83, 36/92, 77399/514, 136/92, 736/82, 77365/103, ‘Réka’, ‘Cleopátra’, ‘Desiree’ és a ‘Boró’	77365/103, 98/91, ‘Cleopátra’, ‘Desiree’ és a ‘Rachel’
Very susceptible	98/91	‘Boró’

Comparing results obtained it is noticeable that some genotypes responded differently to infections by different bacteria species. ‘Rachel’ showed a good resistance against to *Pcc*, while it was moderately susceptible to *Dd*. In contrast, the 98/91 clone was very susceptible to *Pcc* but only moderately susceptible to *Dd*. Moreover, 77399/514 clone was resistant against to *Dd* and ‘Boró’ was the most susceptible to it, both of them were moderately susceptible to *Pcc*.

Classification results of **susceptibility of potato primer tubers** (Vlaslov és Pereverzev, 1989) are summarized in the Table 3.

Table 3. Susceptibility of tubers grown in greenhouse to *Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya dadantii*.

Classification	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	<i>Dickeya dadantii</i>
Resistant	136/92, 77399/514, 'Boró', 'Cleopátra'	'Desiree', 'Boró', 136/92, 77399/514
Moderately resistant	98/91, 'Desiree', 'Rachel'	736/82, 98/91, 77365/103, 'Rachel'
Moderately susceptible	34/85, 77365/103, 736/82	'Réka', 36/92, 34/85
Susceptible	36/92, 1469/83	1469/83
Very susceptible	'Réka'	'Cleopátra'

Comparing bacteria within the method it can be concluded that 'Boró' cultivar and 136/92 and 77399/514 clones were resistant against to both bacteria, while 'Réka' and 1469/83 clone were susceptible to both bacteria.

Summarizing results, it is worth noting, that there are differences and similarities between methods. Since the disease appears on the stem first, it can be supposed, that if the genotype is able to be resistant in its stem-tissue, infection of tubers will also not occur. That is the reason

for we considered the method of infection of *in vitro* shoots to be the most suitable for determination of susceptibility of genotypes.

4.2. Susceptibility of apple genotypes

Results obtained at 5th day after infection are summarized in the table 4.

Table 4. Susceptibility of *in vitro* apple shoots to *Erwinia amylovora* at the 5th day after infection.

Classification	<i>Erwinia amylovora</i>
Resistant	‘Red Fuji’, ‘Freedom’
Moderately resistant	‘Hesztia’
Moderately susceptible	‘Idared’, ‘Húsvéti rozmaring’, ‘Jonagold’
Very susceptible	‘Tenroy’ (Royal Gala)

In general, determination of susceptibility/resistance of apple cultivars is based on the symptoms on shoot and/or flowers. We tested the genotypes just by infection of *in vitro* shoots.

‘Idared’ scion was reported earlier to be very susceptible to fire blight (Sobiczewski *et al.*, 1997; Fischer *et al.*, 2004; Tóth *et al.*, 2005), while in our experiments it was only moderately susceptible.

‘Freedom’ was proven to be resistant, while ‘Tenroy’, ‘Idared’ and ‘Jonagold’ were very susceptible to fire blight in the experiments

conducted at the Wisconsin University (McManus and Heimann, 1997). These results were partly confirmed by our experiments. The ‘Hesztia’ apple cultivar developed by Tóth Magdolna (2012) is one of the state-registered cultivars, which has resistance at multi-level. However, in our experiments it was moderately resistant.

Differences between susceptibility of micropropagated and fully developed plants maybe due to the differences found in their tissue-structure of shoots. Histological differences can be detected between plants grown in *in vitro* comparing to plants grown in field or green house as reported earlier (Jámbor-Benczúr *et al.*, 2001).

4.3. Results of biochemical analysis

We have conducted preliminary experiments in order to ascertain that stress caused by different infection on micropropagated apple and potato plantlets whether can be tracked by measuring of POD activity and carbohydrate content changes or not. Three genotypes with different susceptibility (resistant, moderately susceptible and very susceptible) were tested for each bacterium. Effect of bacteria attacked potato were examined during the first 24 hrs after infection at different times (0; 3; 6 and 24 hrs). In the case of apple the POD activity and carbohydrate content were measured at 0; 6th; 24th; 72nd and 120th hrs. Results obtained from samples collected at 0 hr (immediately after infection) were the control results.

Results of POD activity are summarized in the table 5.

Table 5. Effect of different bacterium species on the change of POD activity.

Observation aspects	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	<i>Dickeya dadantii</i>	<i>Erwinia amylovora</i>
Initial POD values	R>EF	R>EF	R>EF
The time of change occurred after infection	0-6 h ↓ 6-24 h ↑	0-24 h ↑	0-24 h ↓ 24-120 h ↑
Change characters	EQUILIBRATED	PERMANENT	EQUILIBRATED

Clones with different susceptibility showed differences in their initial POD activity. Resistant genotypes showed higher POD activity than the very susceptible genotypes in both plant species. The infection caused changes in POD activity, in such a way, that it decreased significantly at the 6th hr after infection by *Pcc* and then it began rise. However, change induced by infection was not permanent, it was equilibrated. When infection was made by *Dd* the POD activity also increased. The rate of changes was greater in very susceptible genotypes than in the resistant clones. These results were similar to those observed in experiments with *Pcc*. However, these changes were proven to be permanent, and a continuous increase can be supposed.

In the case of apple, changes induced by *Ea* infection could be observed at 24th after infection, when a decreased POD activity could be detected, followed by a raise. Similar results were obtained on fully developed pear plants as reported by Honty (2010).

During carbohydrate content analysis of *in vitro* shoots, glucose and fructose could be detected in potato, while glucose, fructose, sacharose and D-sorbitol could be detected in apple (table 6.).

Table 6. Effect of different bacterium species on the changes of carbohydrate content.

Observation aspects	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	<i>Dickeya dadantii</i>	<i>Erwinia amylovora</i>
Initial carbohydrate content	Glucose: EF>R Fructose: EF>R	Glucose: EF>R Fructose: EF≤R	Glucose: EF>R Fructose: EF<R Saccharose: EF=R Sorbitol: EF>R
The time of change occurred after infection	0-6 h ↓ 6-24 h ↑	0-24 h ↑	24 h
Change characters	EQUILIBRATED	PERMANENT	EQUILIBRATED

Pathological process was characterised well by changes in the fructose content in the potato, and in the sacharose content in the apple. Infection by *Pcc* induced a reduction in the fructose content at first, and then it was equilibrated. In contrast, infection by *Dd* resulted in a

continuous raising in fructose content in both the resistant and the susceptible genotypes. Infection by *Ea* induced opposite trends in the changes of saccharose content in genotypes with different susceptibility. We can conclude, that host plants responded to the different bacterial infections on the same way, thus pathological process does not depend on the pathogens. Biochemical processes induced by biotic stress are the same in both the *in vitro* plantlets and in the whole plant.

4.4. New scientific results

Summarizing new scientific results as follows:

1. We developed a new infection method involved micropropagated shoots and microtubers for evaluation of resistance characters of potato genotypes against to *Erwinia* species inducing soft-rot.
2. We have revealed, that the rate of resistance can reliable be characterised by evaluation of symptoms occurred on *in vitro* shoots, considering methods we have tested.
3. Cultivars and clones were classified according to their susceptibility based on our results obtained on shoots and tubers.
4. We have compared the responses of plants to soft-rot and shoot-wilting based on the changes in the POD stress enzyme activity and carbohydrates at first.

5. We have first demonstrated the suitability of biochemical markers for determination of stress-tolerance ability of genotypes in experiments conducted with micropropagated plantlets.
6. Evaluation methods of *in vitro* potato and apple shoots was complemented by back-isolation of bacteria from infected tissue and counting of developed colonies.

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