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## Effect of *Pseudomonas* spp. on inoculation of young plants of *Fraxinus excelsior* stem with *Diplodia mutila*

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**Abstract:** Bacteria *Pseudomonas aureofaciens*, *P. cepacia* and *P. fluorescens* biovar I was tested against fungus *Diplodia mutila* on stems of 2-year-old plants of European ash in greenhouse experiments. The interaction of bacteria and fungus was assessed on the base of length of necrotic lesions, caused by *D. mutila*, extended from point of inoculation. The strongest antagonistic effect was noted in the case of *P. aureofaciens*.

**Additional key words:** bacteria, fungus, antagonism, in vivo

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### Introduction

*Diplodia mutila* Fr. apud Mont., isolated from apical parts of European ash (*Fraxinus excelsior* L.) shoots showing bark necrosis, was able to induce necrotic lesions following wound inoculation of stems of 2-month-old seedlings of *F. excelsior* in greenhouse conditions (Przybył 2002 a, b). The growth of *D. mutila* mycelium *in vitro* was inhibited by bacteria *Pseudomonas aureofaciens* Kluver, *P. cepacia* Burkh. and *P. fluorescens* Migula – biovar I, and significant differences between bacteria were observed. The poorest growth of *D. mutila* was observed in co-cultures with *P. aureofaciens* (Przybył and Jędrzejowska 2001, Przybył 2002 a).

The aim of the study reported here was to determine the effect of *P. aureofaciens*, *P. cepacia* and *P. fluorescens* I on the inoculation effect of 2-year-old seedlings of *F. excelsior* with *D. mutila*.

### Material and methods

#### Bacterial and fungal material

*Diplodia mutila* and *Pseudomonas* spp. were isolated from dark brown necrotic lesions occurring on the stem bark of several years old plants of *Fraxinus excelsior* in spring 2001 (Przybył and Jędrzejowska 2001, Przybył 2002 a, b).

The cultures of bacteria *P. aureofaciens*, *P. cepacia* and *P. fluorescens* I and the fungus *D. mutila* were stored at +3°C and then transferred onto fresh media and incubated at 23–24°C. The following media were used: for bacteria – King B medium (proteose peptone (Difco Defraud, USA) 2%, glycerol 1%, KH<sub>2</sub>PO<sub>4</sub> 0.15%, Mg SO<sub>4</sub> 0.15%, agar 1.5% distilled H<sub>2</sub>O; pH 6.9); for *D. mutila* – 4.8 % (w/v) malt extract agar (MEA; Merck Darmstadt, Germany; pH 5.5).

## Effect of bacteria on length of necrotic lesions caused by *D. mutila*

*Pseudomonas aureofaciens*, *P. fluorescens* 1, and *P. cepacia* were tested on 2-year-old seedlings of *F. excelsior* growing in pots filled with garden soil and peat (1:1). Twelve seedlings (4 seedlings per replication) per species were used. The surface of plant stems was superficially sterilized before inoculation with cotton wool dipped with 70% alcohol. Then the stems were wounded by removal of bark disc (diameter ca. 5mm) with a razor blade. Two drops of water suspension of tested bacteria ( $10^8$  cfu per 1 ml) were placed on wound twice, at 2h intervals, and then the wounds were covered with autoclaved wet cotton wool and a Parafilm strip. After 24h, a disc of *D. mutila* culture (diameter ca. 5 mm) was placed above, near the bacteria inoculation point. The fungal disc was cut from the margin of a 7-day-old cultures of *D. mutila* grown on MEA.

During the experiment, the plants were kept in a greenhouse with natural light and ambient temperature variation (20–30°C).

In control 1, sterile distilled water was used before inoculation with the fungus. In control 2, the water suspensions ( $10^8$  cfu per 1 ml) of each of the bacteria were used without the fungus.

Observations of necrosis were performed after 2, 4, 7 days and then at weekly intervals for 2 months. Data were expressed as mean lesion length after 2 months. The two-way analysis of variance and Tukey's test

HSD were used (Statistica PL 1997; StartSoft Polska Inc., USA).

Re-isolations of *D. mutila* was attempted after 2 months. The sections (diameter ca 3 mm) were taken from margin of lesions, above and below each inoculation point or from the point of inoculation. Five seedlings for each of the tested bacteria were randomly selected for re-isolation of the fungus.

## Results

### Effect of bacteria on length of necrotic lesions caused by *D. mutila*

Inoculations with *D. mutila* isolate were followed by brownish lesions on which pycnidia of the fungus and superficial wounds, formed on necrotic area, were observed two months after inoculation (Fig. 1). Bark tissues, wood and pith showed brown to nearly black discoloration. Two months after inoculation the mean length of lesions caused by *D. mutila* was 12.7 mm (range 9.5–18 mm). Analogous symptoms were observed on shoots treated with bacteria of *P. fluorescens* I and *P. cepacia*, but pycnidia were absent. The length of external necrotic lesions observed on shoots treated with *P. fluorescens* I and *P. cepacia* were 8.0 mm (range 7–12 mm) and 7.1 mm (range 7–10 mm), respectively. In the case of *P. aureofaciens* the mean length of discoloration was 3.7 mm (range 3–6 mm) (Figs. 2 and 3, Table 1).

*D. mutila* was re-isolated from necrotic sections of control stems and from those inoculated with *P.*



Fig. 1 Symptoms after inoculation with *D. mutila* (point of fungus inoculation – arrow; bar = 5 mm)  
Figs. 2 and 3. Symptoms after inoculations with *P. aureofaciens* and *D. mutila* (point of fungus inoculation – arrow; bar = 10 mm)

Table 1. Effect of *Pseudomonas* spp. bacteria on length of necrotic lesions caused by *D. mutila*

Experiments	Mean lesion length (mm)
<i>Diplodia mutila</i> ; control	12.7 a
<i>D. mutila</i> and <i>P. fluorescens</i>	8.0 b
<i>D. mutila</i> and <i>P. aureofaciens</i>	3.7 c
<i>D. mutila</i> and <i>P. cepacia</i>	7.1 bc

Data followed by the same letters do not differ significantly ( $\alpha < 0.05$ )

*fluorescens* I and *P. cepacia*. In the case of *P. aureofaciens* inoculation, *D. mutila* was re-isolated only from the points of inoculation.

No lesions developed on any of the control seedlings inoculated only with bacteria (control 2).

## Discussion

Among bacteria that are able to inhibit the activity of pathogenic fungi, *Pseudomonas* spp. are of special significance (Upadhyay, Jahaswal 1992, Brooks et al. 1994, Pedersen et al. 1999, Przybył and Żłobińska-Podejma 2000). As an instance, *P. putida* was most effective at inhibiting mycelial growth of *Piptoporus betulinus* *in vitro*, whereas *P. aureofaciens* and *P. fluorescens* biovar 1 were less active (Przybył and Żłobińska-Podejma 2000). *Pseudomonas cepacia* is known to be antagonistic towards many pathogenic fungi, e.g. *Sclerotinia sclerotiorum* (Upadhyay and Jahaswal 1992).

In an earlier *in vitro* study, *P. aureofaciens* and *P. cepacia* significantly inhibited the growth of *D. mutila* mycelium after 2, 7 and 14 days of incubation in comparison with control. *P. fluorescens* biovar I had no effect on the growth of the fungus after 2 days of incubation. In the case of *P. cepacia* and *P. fluorescens* I no significant differences in fungus growth were observed between 7 and 14 days of incubation (Przybył and Jędrzejowska 2001).

The *in vivo* inoculation test on stems of 2-year-old seedlings of *F. excelsior* showed the antagonistic effect of *P. aureofaciens* towards *D. mutila*. Antagonistic activity of *P. fluorescens* I and *P. cepacia* is doubtful. Length of necrotic lesions caused by both bacteria species significantly differ from the control data (*D. mutila* inoculation), but no visible macroscopic differences in symptoms were observed between them.

Generally, to our knowledge there is little information available in literature on interaction between *D. mutila* and bacteria or fungi. In the latter case, Moricca et al. (1993) and Ragazzi et al. (1996) found antagonistic activity of *Acremonium mucronatum* towards *D. mutila* both *in vitro* and *in vivo*.

The importance of *P. aureofaciens* for biological control of *D. mutila* remains uncertain. Pukacki and Przybył (1999) found that *P. aureofaciens* isolated from

wood of *Betula pendula* can increase frost sensitivity of plants. It is plausible that *P. aureofaciens* obtained from European ash had the same feature. However, it seems likely that the antagonistic bacteria can affect the frequency of *D. mutila* occurrence in nature by inhibiting mycelial growth and spore germination.

The results obtained in these preliminary studies provide encouragement for more extensive studies in this direction.

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