Siderophores are the Main Determinants of Fluorescent *Pseudomonas* Strains in Suppression of Grey Mould in *Eucalyptus urophylla*

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**Abstract:** Three fluorescent *Pseudomonas* strains and their pseudobactin siderophore-minus mutants were investigated in suppression of eucalypt grey mould caused by *Botrytis cinerea*. Results from *in vitro* antagonistic tests showed that *Pseudomonas fluorescens* WCS374r and *P. putida* WCS358r inhibit mycelial growth of *B. cinerea* by competition for iron. When WCS358r, WCS374r and WCS417r were applied to wounded leaves 10 h prior to the inoculation of pathogen, they suppressed eucalypt grey mould, and reduced the percentage of necrotic spots on leaves by 48.9%, 58.3% and 40.3%, respectively. Strains WCS358r and WCS374r were effective as well when the mixture of either strain and pathogen was applied to wounds on leaves. However, if three strains were applied to wounds 12 h or 24 h posterior to inoculation of pathogen, the control effects decreased sharply. The pseudobactin-minus mutants of WCS358r, WCS374r and WCS417r partially or fully lost their suppressive ability of the disease. These results demonstrate that siderophores are important bacterial determinants in suppression of eucalypt grey mould.

**Key words:** biological control; *Botrytis cinerea*; *Eucalyptus urophylla*; *Pseudomonas*; siderophores

荧光假单胞杆菌的嗜铁素是控制桉树灰霉病的主要因子

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**Abstract:** 本文对3个假单胞杆菌菌株（*Pseudomonas* spp.）及其嗜铁素（pseudobactin siderophore）缺失突变体防治桉树灰霉病进行了研究。平板拮抗活性测定表明，荧光假单胞杆菌（*P. fluorescens*）WCS374r 菌株和恶臭假单胞杆菌（*P. putida*）WCS358r 菌株通过抑制铁离子的竞争抑制灰霉菌的生长。在接种灰霉菌菌之前 10 h 将 WCS358r、WCS374r 和 WCS417r 施用于受伤的桉树叶片后，可分别降低发病率 48.9%、58.3% 和 40.3%；将 3 种生防菌分别与灰霉菌菌混合后接种桉树叶片，WCS358r 和 WCS374r 仍然能够显著地降低发病率；在接种灰霉菌 12 h 后再施用生防菌，WCS358r 和 WCS374r 对病菌仍具有一定的抑制作用，而在 24 h 后施用生防菌，3个菌株均未表现显著的防治效果。WCS358r 和 WCS417r 的嗜铁素缺失突变体虽然还能有效地防治灰霉病，但与 WCS374r 相比，防病效果减

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**Biography:** RAN Long-xian (1962-), male, a native of Guizhou, professor in Forest Pathology, Ph. D., graduated from Utrecht University, The Netherlands, and mainly involved in biological control of plant diseases; E-mail: longxiangran@163.com.
Eucalypt grey mould caused by *Botrytis cinerea* Pers. ex Fr. is a severe epidemic in eucalypt nurseries of south China. It mainly infects the seedlings of *Eucalyptus urophylla* S. T. Blake and *E. grandis* × *E. urophylla*, and in some nurseries, mortality of seedlings reached 10%–40%. Chemical fungicides were widely applied for the control of this disease. However, there has been a report on development of resistance of *B. cinerea* populations to benzimidazoles, leading to efforts to develop alternative measures for disease management. Several saprophytic bacteria, fungi and yeasts have been reported to be effective against grey mould in several crops.

Siderophores are low-molecular-weight molecules that are secreted by microorganisms to take up iron from the environment. They have been shown to play a role in plant growth promotion, suppression of several plant diseases, induction of systemic resistance, and have relations to virulence mechanisms in microorganisms pathogenic to both animals and plants. However, there were reports in which siderophores had less effects in suppression of plant diseases. For example, the fluorescent siderophore secreted by *Pseudomonas fluorescens* strain 2-79 was not responsible for the protection against “take-all” of wheat root disease, nor in *P. fluorescens* strain CHA0 against black root rot of tobacco.

*P. putida* WCS358, *P. fluorescens* WCS374 and WCS417 all produce pseudobactin-type siderophores, and they have been extensively investigated for their suppressive activities against different plant pathogens. This study evaluates the role of siderophores in suppression of eucalypt grey mould by these fluorescent *Pseudomonas* spp. strains.

## 1 Materials and Methods

### 1.1 Microbial cultures and inocula

The sources and relevant characteristics of the microbes used are listed in Table 1.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Botrytis cinerea</em> CNS03</td>
<td>Isolated from naturally infected seedling of <em>E. urophylla</em> in Xijiang Forestry Bureau, Guangdong, China</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em> WCS374r</td>
<td>rif' strain of WCS374 isolated from potato rhizosphere; amp', chl', rif'</td>
<td>[7, 12]</td>
<td></td>
</tr>
<tr>
<td>JM374</td>
<td>Tn5 mutant of WCS374, sid'; amp', chl', Kan'</td>
<td>[13]</td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em> WCS417r</td>
<td>rif' strain of WCS417 isolated from wheat rhizosphere; amp', chl', rif'</td>
<td>[14, 15]</td>
<td></td>
</tr>
<tr>
<td>S680</td>
<td>Tn5 mutant of WCS417, sid'; amp', chl', Kan'</td>
<td>[14]</td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> WCS358r</td>
<td>rif' strain of WCS358 isolated from potato rhizosphere; amp', chl', rif'</td>
<td>[12, 16]</td>
<td></td>
</tr>
<tr>
<td>JM218</td>
<td>Tn5 mutant of WCS358, sid'; amp', chl', Kan'</td>
<td>[17]</td>
<td></td>
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</tbody>
</table>

* Abbreviations: sid = pseudobactin siderophore; amp', chl', Kan', rif' = resistant to ampicillin, chloramphenicol, kanamycin, and rifampin, respectively.
The pathogen strain, B. cinerea CN503 was cultured to sporulation on PDA agar plates at 23 – 25°C for one week. Spores were washed with sterile distilled water containing 0.01% household detergent. After removing mycelial debris, the spore concentration was determined and the inoculum was adjusted to $5 \times 10^3$ spores/mL.

### 1.2 In vitro antagonism by fluorescent pseudomonads against B. cinerea

*In vitro* antagonism between *Pseudomonas* spp. and *B. cinerea* was studied on KB agar plates. One hundred microlitre of spore suspension of *B. cinerea* containing $10^6$ spore/mL was spread on the plate (6 cm diameter). Immediately thereupon, a 7 mm diameter agar disk cut from a plate on which one of the *Pseudomonas* strains had been grown for 24 h at 28°C, was placed on the center of the plate. Zones of inhibition of *B. cinerea* growth were measured after incubation for 72 h at 23 – 25°C. Effects of iron on *in vitro* antagonism between pseudomonads and *B. cinerea* were studied on KB plates with 200 μmol/L FeCl₃.

### 1.3 Assays for biological control of B. cinerea

When the seedlings of *E. urophylla* reached the height of 10 – 12 cm, leaves were pressed gently on their surfaces with a round stainless steel rod (2 mm diameter), resulting in 2 wounds per leaf. Three pairs of leaves for each seedling were wounded and 10 seedlings were used for each treatment. Three kinds of bioassays were performed as follow; 1) The wound was immediately covered with 10 μL of bacterial suspension at $10^6$ cfu/mL or 10 mmol/L MgSO₄ as a control. The treated seedlings were kept in a cabinet at a temperature of 23 – 25°C and a relative humidity of 70%. When the drops of bacterial suspension were air-dried about 10 h later, each wound was inoculated with 3 – 5 μL of *B. cinerea* at $5 \times 10^3$ spores/mL. 2) *Pseudomonas* strains ($10^6$ cfu/mL) or 10 mmol/L MgSO₄ as a control were mixed with the pathogen ($5 \times 10^3$ spores/mL) in the ratio of 2:1 (v/v). Ten microlitre of the mixture was applied to each wound on the leaves. 3) The wounded leaves were inoculated with 3 – 5 μL of pathogen ($5 \times 10^3$ spores/mL), and the bacterial suspension ($10^8$ cfu/mL) was applied to the infected wounds 12 h or 24 h later.

The inoculated plants were then transferred to a container covered with plastic film and placed in a cabinet with a 12 h light and 12 h dark cycle at 23 – 25°C for disease development. The number of necrotic and spreading spots on the leaves was scored 3 d or 4 d later. Each bioassay was repeated at least three times.

### 1.4 Data analysis

The percentage of spots with dark brown and spreading necrosis was determined for each plant. Means of treatments were statistically compared using one-way analysis of variance (ANOVA), followed by Fisher’s test for least significant differences at $\alpha = 0.01$ or 0.05.

### 2 Results

#### 2.1 Antagonistic activity in vitro

*P. putida* strain WCS358 and *P. fluorescens* strains WCS374 and WCS417 have been used extensively in studies on biocontrol of *Fusarium* wilt of carnation, radish and tomato, and of bacterial speck in *Arabidopsis*. Both strains WCS358r and WCS374r suppressed mycelial growth of *B. cinerea* by competition for iron, evidenced by the complete loss of inhibitory activity in the presence of 200 μmol/L FeCl₃. The pseudobactin-minus mutant of WCS358r, JM218 did not inhibit mycelial growth of *B. cinerea*. However, a pseudobactin-minus mutant of WCS374r, JM374, maintained the ability to inhibit growth of *B. cinerea*. For strain WCS417r, no clear inhibition zone was observed, but mycelial growth was reduced, whereas its pseudobactin mutant S680 did not reduce mycelial growth (Table 2).
Table 2  In vitro growth inhibition of B. cinerea by Pseudomonas strains and their pseudobactin-minus mutants on KB agar plates with or without 200 μmol/L FeCl₃

<table>
<thead>
<tr>
<th>Media</th>
<th>Strains</th>
<th>Strains</th>
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<tbody>
<tr>
<td></td>
<td>WCS358r JM218</td>
<td>WCS374r JM374</td>
</tr>
<tr>
<td>KB (Fe⁺)</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>KB (Fe⁺)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: a = the inhibition zone includes diameter of the agar disk; b = no inhibition zone around the agar disk was observed; c = no clear inhibition zone observed, but growth reduction obvious; n. d. = not determined. The inhibition zone is given in mm.

2.2 Suppression of grey mould by Pseudomonas spp.

Three strains were tested for their abilities to control B. cinerea in vivo by applying them to wounds 10 h prior to inoculation of pathogen. WCS358r, WCS374r and WCS417r all protected eucalypt seedlings against B. cinerea and significantly reduced the proportion of brown and spreading spots by 48.9%, 58.3% and 40.3%, respectively (Fig. 1).

Fig. 1  Suppression of grey mould by three Pseudomonas strains which were applied to the wounds 10 h prior to inoculation of pathogen

CK = treated with 10 mmol/L MgSO₄. Bars marked by different letters indicate statistically significant differences between treatments (Fisher’s LSD test; α = 0.01)

Based on the result of the first assay, three strains were tested further by mixing each of them with the pathogen (Fig. 2), or applying them after the pathogen was inoculated to the wounds for 12 h or 24 h (Fig. 3), respectively. The result of the former bioassay was shown in Fig. 2. Both WCS358r and WCS374r were able to significantly reduce the percentage of necrosis by 46.5 and 16.7, respectively (α = 0.01), in which WCS358r was the best strain to suppress grey mould, whereas WCS417r was not. However, in the latter bioassay the control effect decreased largely when the infected wounds were applied with bacterial suspension 12 h later, though strains WCS358r and WCS374r still significantly reduced the proportion of necrotic and spreading spots (α = 0.05) in Fig. 3. When the bacterial strains were applied to the inoculated wounds with pathogen 24 h later, none of them had protection against grey mould, indicating that these strains could not prevent infection or spread of germinated spores or mycelia of B. cinerea in the tissues of eucalypt leaves (Fig. 3).

Fig. 2  Suppression of grey mould by Pseudomonas strains (10⁶ cfu/mL) which were mixed with the pathogen (5 × 10⁵ spores/mL) in the ratio of 2:1 (v/v)

CK = treated with 10 mmol/L MgSO₄. Ten microlitre of the mixture was applied to each wound on the leaves. Bars marked by different letters indicate statistically significant differences between treatments (Fisher’s LSD test; α = 0.01)
3 Discussion

WCS358r and WCS374r inhibited mycelial growth of *B. cinerea* *in vitro* by competition for iron. However, a pseudobactin-minus mutant of WCS374r did still inhibit mycelial growth. This could be explained by the production of a second siderophore, pseudomonine, in strain WCS374\(^{10}\).

The suppression effects of *Pseudomonas* strains over grey mould in *E. urophylla* were strongly affected by application methods. When the biological strains were applied prior to inoculation of pathogen, all three fluorescent *Pseudomonas* spp. strains tested controlled eucalypt grey mould *in vivo*. If the mixture of biological strains and pathogen were dripped to the wounds, only strains WCS358r and WCS374r had control effects. When the seedlings were inoculated with pathogen followed by application of bacterial strains 12 h later, WCS358r and WCS374r were able to suppress grey mould. However, if the time interval between inoculation of pathogen and application of bacterial strains was over 24 h, none of them showed suppressive effects anymore.

The pseudobactin-minus mutants of WCS358 and WCS417 fully lost their disease suppressive abilities compared with their parental strains, suggesting that an important role of pseudobactin-type siderophore in suppression of grey mould by these two strains. However, the pseudobactin-minus mutant of...
strain WCS374 was capable of suppressing the disease as well, indicating the second siderophore, pseudomonine, produced by WCS374 was partly responsible for the disease suppression.

Based on the results, a conclusion can be drawn that inhibition of mycelial growth of B. cinerea in vitro is positively related to disease suppression in vivo and these three fluorescent Pseudomonas strains suppressed eucalypt grey mould by competition for iron in the tested bioassay. These results are essentially consistent with what were obtained in carnation and radish[14,16]. In radish, the pseudobactin-type siderophore in strain WCS358 was responsible for suppression of Fusarium wilt, whereas in strains WCS374 and WCS417, both the wild-type and pseudobactin-minus strains suppressed Fusarium wilt[14], demonstrating that pseudobactin-type siderophores of these two strains were less effective in suppression of Fusarium wilt. Since siderophores have been implicated in induction of systemic resistance[29], in the E. urophylla-B. cinerea model the mode of action of siderophores in disease control will be further investigated.

References


[16] Leeman M, den Ouden F M, van Pelt J A, et al. Suppression of fusarium wilt of radish by co-inoculation of...


