Effectiveness of Bacterial and Fungal Isolates to Control *Phoma lingam* on Oilseed Rape *Brassica napus*

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ABSTRACT

Blackleg disease caused by *Phoma lingam* is an important disease of oil seed rape (*Brassica napus*) causing losses up to 95%. The efficacy of microbial antagonists against *P. lingam* in greenhouse was tested. *Serratia plymuthica* HRO-C48 and *Gliocladium catenulatum* J1446 were able to reduce the disease intensity of OSR cotyledones infested with *P. lingam* at rates 44% and 52% respectively. The reduction of the infestation of the root collar in BBCH14/15 was evaluated as well. Plants treated with a suspension of the antagonists (2 × 10⁵ cfu/plant) and inoculated with either pycnidiospore suspension (2 × 10⁷ cfu/ml) or agar disks grown with *P. lingam* mycelium, showed a reduced infestation rate of 53% - 93% in the presence of *S. plymuthica* and 46% - 77% in the presence of *G. catenulatum*. The efficacy of the antagonist depends highly on their concentration inside OSR seeds. Below 10⁵ cfu/seed no significant difference was recorded between control untreated and infested plants.

Keywords: Biological Control; Oilseed Rape; *Phoma lingam*; Bacteria

1. Introduction

Oilseed rape (OSR) *Brassica napus* is a major crop grown for its oil in many parts of the world including Canada, India, China and Europe [1]. In favourable conditions, *B. napus* is considered to have the highest yield potential of all *Brassica* oil crops [2]. Winter and spring varieties of the crop exist. Winter varieties are mainly grown in Europe and China and spring varieties in areas such as Australia and Canada. Winter OSR is an excellent starter crop in cereal rotations, due to its positive impact on disease control and soil improvement. Its cultivation favours the minimum tillage which is of great interest for sustainable agriculture [3].

In the last 20 years, an increase of 260% in OSR production has been recorded worldwide, driven by the enormous economic significance of oilseed crops as a renewable raw material used for a variety of applications in food and non-food areas [4]. In Germany, OSR is the most important oilseed crop, with acreage of more than 1.3 million hectares in 2005. Yields ranged between 2.9 and 3.5 tons per hectare. In 2005, 5.1 million tons of rapeseed was harvested approximating a total seed-oil yield of 2 million tons [3].

With the increased production of oilseed rape (OSR) *B. napus* spp. *napus*, reports on pathogen causing diseases have increased simultaneously. Oilseed rape however, is susceptible to many fungal pathogens such as *Pyrenopeziza brassicae* Raw. (anamorph *C. concetricum* Grev.), *Leptosphaeria maculans* (Desm.) Ges. Et De Not. (anamorph *Phoma lingam* (Tod.) Desm.), *Sclerotiorum* (Lib.) de Bary and *Verticillium dahliae* Kelb. Stem canker (also termed blackleg) caused by *P. lingam* is of major economic importance in the main OSR growing areas of Australia, Canada and Europe [5]. In some instances (e.g., when chemical fungicidal applications are not effective) heavy losses, as much as 95% might be caused by these fungi [1]. In Europe, the severity of blackleg disease was reported to be significant since 1966 [6]. The disease epidemic differs greatly between seasons, regions and crops. In some instances basal phoma stem canker can potentially cause total crop loss [5,7].

Fungicides are the most effective means for controlling stem canker, but their use must be optimised to achieve maximum economic response and to avoid unnecessary fungicide applications [8]. Beside the side effects to the environment and development of fungicide resistance, use of foliar fungicides in association with cultivars with little or no resistance has proved to be ineffective for controlling phoma stem canker, because the current fungicides have low eradicant activity and are effective as protectants for only a short period [5]. Biocontrol methods
might be promising alternatives to reduce the use of fungicides. Various microbial antagonists, including strains of bacteria of the genera \textit{Bacillus}, \textit{Pseudomonas} and \textit{Serratia} and strains of fungi such as \textit{Gliocladium} and \textit{Trichoderma}, have shown biocontrol activity against damping-off diseases and \textit{Botrytis} on \textit{Photinia}, in conventional \textit{Brassica} seedling [9].

The aim of this work is to test the efficacy of different biocontrol antagonistic agents (BCA) against the Black leg disease in OSR.

2. Materials and Methods

2.1. Cultivation and Maintenance of Antagonistic Biocontrol Agents

Bacterial isolates \textit{Serratia plymuthica} HRO-C48, \textit{Bacillus subtilis} B2g, \textit{Pseudomonas fluorescens} E9, 1Re2-6, \textit{Paenibacillus polymyxa} 1P1-2 and \textit{Pseudomonas chlororaphis} MA342 were grown each in 250 ml Erlenmeyer flasks containing 50 ml of 20 g/l tryptic soy broth (TSB) (Difco Laboratories, USA) supplemented with 100 µg/ml refampicin. The flasks were placed on a rotary shaker (180 rpm) for 1 week. Aliquots of 700 µl of each bacterial isolate were transferred into Eppendorf tubes containing 300 µl of 50% sterile glycerol. The cultures were stored after mixing at –80°C.

The fungus \textit{Gliocladium catenulatum} isolate J1446 (commercially produced under the name Prestop® (Ververda, Finland) was grown on Malt extract agar (MEA) media containing 100 mg/l streptomycin and 50 mg/l red Bengal. Cultures were incubated at 22°C for one week. For spore production, MEA agar disks containing fungal mycelium were grown in 250 ml Erlenmeyer flasks containing 50 ml malt extract liquid media on a rotary shaker (140 rpm) for two weeks at 20°C. Spore concentration of the fungus was then adjusted to 6 × 10^8 cfu/ml in 0.85% normal saline.

2.2. \textit{In Vitro} Antagonistic Tests

Antagonistic activity of the biocontrol agents against \textit{P. lingam} was determined using dual culture technique. 100 µl of \textit{P. lingam} spore suspension (2 × 10^7 Spore/ml) were spread on plates containing V8 agar media. Each antagonist was then streaked on the plate (3 streaks/plate) and incubated either at 10 or 22°C for 7 days. The control experiments were done by using sterile distilled water instead of antagonists. The effect of each BCA was determined by measuring the inhibition zones of fungal growth. The rating scale was: 4, inhibition zone > 10 mm; 3, inhibition zone 5-10 mm; 2, inhibition zone < 5 mm; 1, growth was stopped at the bacterial-streak line; 0, no inhibition zone and often \textit{P. lingam} was growing over the bacterial streak [9]. The experiment was repeated using agar disks grown with the fungus instead of fungal spores. Two V8 agar disks (7 mm diameter) grown with 7 days old \textit{P. lingam} were placed about 20 mm from each of BCA streak. The plates were further incubated as mentioned above and zones of inhibition were determined according to the above scale.

To test the effect of \textit{G. catenulatum}, WA agar disks grown with the fungus were used instead of bacteria.

2.3. Inoculation of Cotyledons with \textit{P. lingam}

Cotyledons were punctured with a needle and 10 µl of pycnidiospore suspension (2 × 10^7 Spore/ml) was placed on the wound as described by Mithen et al. (1987) [10]. Control plants were inoculated with 10 µl sterilized distilled water. Plants were further incubated in growth chamber cabinets for 14 days. To prevent plant proliferation, growing leaves were removed every two days during the experimental time. Disease incidence was then measured according to Kutcher et al. (1993) [11] in a rating scale from 0 - 6 as follows: 0: no symptoms; 1: lesions on the infection site < 1.5 mm; 2: lesions on the infection site 1.5 - 3.5 mm; 3: lesions on the infection site > 3.0 mm; 4: gray to green tissue collapse 3.1 - 5.0 mm; 5: gray to green tissue collapse > 5.0 mm (≤10 pycnidia); 6: gray to green tissue collapse > 5.0 mm (>10 pycnidia) [12].

2.4. Inoculation of OSR Plantlets with \textit{P. lingam}

The efficacy of the biocontrol agents on disease development at an earlier stage of plant growth was also tested. For this, OSR plantlets at growth stage of BBCH 14/15 [13] were inoculated at the stem base either with V8 agar disks (7 mm diameter) grown with \textit{P. lingam} or with 40 µl pycnidiospors (2 × 10^7 spore/ml). Plants were incubated for 50 days in growth chamber under the above mentioned conditions. Infestation with \textit{P. lingam} was then determined according to Kutcher et al. (1993) [14]. The volume of disease tissue (VDT) was assessed in a rating scale of 0 - 4 as illustrated in Table 1.

2.5. Colonization Studies

To study the population size of the antagonists in OSR rhizosphere, seeds were treated with the antagonists by soaking 1 g of seeds in 1-ml bacterial suspension for 5 h at 20°C. The seeds were grown in growth chambers at 22°C ± 1°C and 75% ± 5% relative humidity. After different periods of time (1, 10 and 30 days), 5 g of roots with adhering soils from five OSR seedlings of each treatment were placed in an Erlenmeyer flask containing 40 ml sterile water and shaken for 1 h at 24°C. Serial dilutions (10^-2, 10^-3 and 10^-4) were prepared and aliquots (100 µl) were spread on TSA media supplemented with 150 mg/ml refimpicin. Plates were further incubated at 25°C for 48 h and the number of bacteria was estimated as log cfu/g root fresh weight.
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2.6. Field Experiments

Experiments were conducted in naturally infested fields with *P. lingam*. The field which was located in Achterwehr, (54°19'N, 9°58'E) is owned by the institute of agronomy and crop science, Christian Albrecht University of Kiel. OSR was cultivated in a Randomized Block Design in which four blocks were used as replicates. The area of each block was 14 × 3 m and divided into smaller areas (4.5 × 3 m each).

Before sowing, OSR seeds were treated with the antagonists as follows: *S. plymuthica*, HRO-C48Rif, *S. plymuthica*, HRO-C49Rif combined with the fungicide Metconazol (applied two weeks after starting the experiment), *G. catenulatum* and *G. catenulatum* combined with the fungicide Metconazol (applied two weeks after starting the experiment). Untreated seeds were used as control. Seed coating with insecticides and fungicides was achieved as follows: seeds were first treated with insecticide Chinook was added (20.0 ml/kg seeds), followed by adding the fungicide DMM (Dimethomorph) (10 g/kg seeds). While rotating at 400 rpm, talcum blue was added until all liquid is bound to the seeds. The seeds were then dried under laminar flow for 1 h. The disease severity was assessed by counting the number of pycnidiospores on OSR leaves.

Population size of *S. plymuthica*, HRO-C48 and *G. catenulatum* in OSR rhizosphere was studied under field conditions. 5 g of roots with adhering soils from five OSR seedlings of each treatment were placed in Erlenmyer flasks containing 40 ml sterile water and shaked for 1 h at 24°C. Serial dilutions (10⁻², 10⁻³ and 10⁻⁴) were prepared and aliquots (100 μl) were spread on TSA media supplemented with 150 μg/ml rifampicin. Plates were further incubated at 25°C for 48 h and the number of bacteria was estimated as log cfu/g root fresh weight.

2.7. Statistical Analysis

Statistical analyses were done using XLStat program (Adinosoft). Analysis of variance (ANOVA) and significant differences for means were calculated after Tukeys HSD test at (P ≤ 0.05).

3. Results

3.1. *In Vitro* Inhibition of Mycelial Growth of *P. lingam*

All of the antagonistic isolates reduced the mycelial growth as well as the growth of pycnidiospores of *P. lingam* on V8 media. The size of the inhibition zone varied according to antagonist and type of media used (Table 2). *G. catenulatum* was the most effective in inhibiting the mycelial growth of *P. lingam* on both types of media at 22°C and caused a very strong inhibition zones >10 mm. However, its effect on pycnidiospores was lower (<5 mm). Except for *B. subtilis*, all bacteria isolates were able to inhibit *P. lingam* since the bacterium did not grow at 10°C. As shown in Table 2 only the isolate *P. fluorescens* (E9) caused week inhibition zone (<5 mm) on both media and temperatures. The effect of *P. chlororaphis* at 22°C on V8 media was more pronounced in inhibiting the growth of pycnidiospores than that at 10°C (inhibition zone >10).

3.2. Effect of Antagonists on Cotyledon Infestation with *P. lingam*

The antagonistic isolates *S. plymuthica*, *P. chlororaphis* were the most effective in reducing the infestation of cotyledons of OSR cultivar Talent grown under growth chamber conditions, and were able to reduce the disease infection by 43.3% (Figure 1). In addition to that, the number of healthy plants of cultivar Talnet under growth chamber at 22°C was significantly higher in the seeds treated with the isolate *P. chlororaphis* (Figure 1). Treatment with *G. catenulatum* resulted in a significant reduction of disease intensity (51.6%) compared to untreated control seeds.

Table 2. *In vitro* inhibition of *P. lingam* mycelium and pycnidiospores grown on V8 and WA media at 10 and 22°C.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mycelia</th>
<th>Pycnidiospores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V-8</td>
<td>WA</td>
</tr>
<tr>
<td></td>
<td>22°C</td>
<td>10°C</td>
</tr>
<tr>
<td><em>S. plymuthica</em></td>
<td>3 3 3 3</td>
<td>3 2 2 2</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>4 0 4 0</td>
<td>4 0 0 0</td>
</tr>
<tr>
<td><em>Pa. polymyxa</em></td>
<td>3 2 2 3</td>
<td>3 3 3 3</td>
</tr>
<tr>
<td><em>P. fluorescens</em> (1Re2-6)</td>
<td>2 3 3 4</td>
<td>4 4 4 4</td>
</tr>
<tr>
<td><em>P. chlororaphis</em></td>
<td>4 4 4 4</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td><em>G. catenulatum</em></td>
<td>4 3 4 3</td>
<td>1 1 1 1</td>
</tr>
<tr>
<td><em>P. fluorescens</em> (E9)</td>
<td>1 2 1 1</td>
<td>2 2 2 2</td>
</tr>
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Figure 1. Effect of seed biopriming with different antagonists on *P. lingam* intensity on cotyledon of OSR cultivars Talent grown in growth chamber at 22°C. Disease intensity (scale 0-6) and the percent of healthy plants were recorded after inoculating the cotyledons 2 × 10⁵ cfu/plant of a spore suspension of 2 × 10⁷ cfu/ml of *P. lingam*. Results of different letters are significantly different after Tukey HSD test at *P* < 0.05, *n* = 45 plant per treatment).

The results showed a pronounced increase in the percent of healthy plants of OSR in the presence of *P. chlororaphis*. In seeds treated with *S. plymuthica* or *G. catenulatum* the percent of healthy plants was higher than that in seeds treated with the other antagonists (Figure 1).

### 3.3. Efficacy of Antagonists on *P. lingam* Infection on Stem Base

Data in Figure 2 shows the effect of the BCAs on reduction of root collar infection. The volume of diseased tissue (VDT) in plants infested with spore suspension of the pathogen was significantly lower (*P* < 0.05) in OSR seeds treated with *S. plymuthica*, *P. chlororaphis* and *G. catenulatum* (1.14 ± 0.8, 1.08 ± 0.4 and 1.09 ± 0.7, respectively). In the presence of *P. fluorescens* the VDT (1.81 ± 0.7) did not differ significantly from the control untreated plants (VDT = 2.04 ± 1.1). In OSR plants infested with V8 agar pieces grown with *P. lingam*, the antagonists reduced significantly the VDT. The effect *P. fluorescens* was lower (but not significantly different) than the effects of the other antagonists (Figure 2).

### 3.4. Colonization of the Rhizosphere with *S. plymuthica* and *G. catenulatum*

Our results showed that the isolates *S. plymuthica*, *P. chlororaphis* and *P. fluorescens* are rhizosphere competent antagonist since they were able to colonize the rhizosphere of ORS in a short period of time. Root colonization was also recorded after seed treatment with *B. subtilis* und *P. polymyxa* (2 - 1.2 × 10⁶ cfu/ml). However, the colonization was lower than that of *S. plymuthica*, *P. chlororaphis* and *P. fluorescens*. After 8 day of plant growth under greenhouse conditions, the average number of bacteria was 1.2 × 10⁵ cfu/g root fresh weight. At the end of the experiment (30 days), colonization rate was 5.2 × 10⁴ cfu/g root fresh weight (Figure 3).

### 3.5. Efficacy of Antagonists against *P. lingam* under Field Conditions

In plants treated with the antagonists, the disease intensity was lower than that of the control untreated plants. As shown in (Figure 4) the average number of pycnidiospore after two weeks of planting was 21.3 pycnidia/plant in the control untreated plants and 2.5 and 1.5 pycnidia/plant in plants treated with *S. plymuthica* and *G. catenulatum* respectively. The combined treatments of *S. plymuthica* and *G. catenulatum* with the fungicides resulted in a pronounced reduction of disease infestation (0.0 and 0.9 pycnidiospore/plant respectively).

The highest number of pycnidiospores was recorded after two weeks of fungicide application. After that the disease intensity decreased and no significant differences were recorded between the numbers of pycnidiospores in control plants and in plants treated with the antagonist or the antagonists combined with the fungicide.

Six months after sowing, the disease intensity was reduced and one month later no disease on leaves was recorded. The disease intensity was significantly lower in treated seeds compared to the control untreated seeds.
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Figure 3. Comparison of root colonization of OSR roots by different antagonists. Data are the means (±SD) of 5 plants taken after 1, 10 and 30 days of incubation under growth chamber cabins at 22°C and 12 h day/night regimes. Seeds were bioprimed with the antagonists by soaking the seeds in bacterial suspensions (2 × 10^10 cfu/ml) for 5 h and dried at room temperature for two days.

Interestingly, no significant difference in disease intensity was seen when the fungicide was combined with the biocontrol agents.

The disease incidence was also calculated at two time points (03.11 and 15.12). In control plants infested with *P. lingam* the disease incidence was 74% and 68% and in plants treated with the *S. plymuthica* combined with the fungicide the disease incidence was 12.5% and 25%.

4. Discussion

In this study, several antagonistic microorganisms including bacteria and fungi were tested for their efficacy against the black leg disease caused by *P. lingam* on oilseed rape. The first step toward successful selection of effective biocontrol agents was done by testing the effect of the antagonists against the pathogen in dual culture assays. As revealed from the dual culture assays, the biocontrol agents differ in their efficacy against *P. lingam*. The size of inhibition zone varied according to the incubation temperature. Although no general relationship exist between the ability of a microorganism to inhibit a pathogen *in vitro* and to suppress disease caused by that pathogen *in planta* [15,16], some authors [17,18] have shown that the antagonistic efficacy might be correlated with the *in vivo* effect of the antagonists. It was found that the results of dual culture assay depend highly on culture media [19,20]. In this work the size of the zone of inhibition caused by the biocontrol agents was affected by the type of media used (V8 and WA media). Because the pathogen *P. lingam* infects mostly winter rape at low temperatures (10°C) the effect of temperature on antagonism was also tested. Except for *B. subtilis* and *P. polymyx* most isolated with high efficacy at 22°C were also able to inhibit mycelial growth at 10°C.

*P. lingam* is able to infect all parts of OSR at all stages of plant development [21]. For example an infection in autumn might cause later an infection of root collar and stem base [22]. The fungus is able to spread from the infected leaves to other parts of the plant causing the disease symptoms [23]. For this reason inhibition of the fungus on cotyledons was studied.

To test the effect of the pathogen on emerging leaves, the leaves of the OSR plants in the physiological growth stage BBCH1-12 were injured mechanically by a sterile needle. A spore suspension was then spread on the wounds and the disease symptoms were monitored after 14 days [11,24]. In *ad planta* tests, more than 50% reduction of the disease caused by *P. lingam* on leaves of OSR seedling grown in growth chamber was recorded in seeds treated with *S. plymuthica*, *P. chlororaphis*, *P. fluorescens* und *G. catenulatum*. The percent of healthy plants increased significantly from less than 10% to 40%- 50% in the presence of the antagonists. However, the efficacy of the gram positive isolates was lower than that of the gram negative bacterial strains.

The isolate *S. plymuthica* was the most effective in controlling the disease caused by *P. lingam* on root collar and stem base. The disease intensity was reduced by 54% and 63% in plants grown in greenhouse and growth chamber conditions. In seeds treated with *G. catenulatum*, *P. chlororaphis*, *P. fluorescens* and *G. catenulatum* the percent of healthy plants increased significantly from less than 10% to 40% - 50% in the presence of the antagonists. However, the efficacy of the gram positive isolates was lower than that of the gram negative bacterial strains.

For effective protection against plant pathogens, the antagonist must be able to colonize successfully the rhizosphere of the plant [25]. The antagonists must compete with other microorganisms in the root system of the plants in order inhibit the attack of pathogens [26]. It was found that colonization patterns differ between different antagonistic microorganisms [27]. In our experiments,
OSR roots were highly colonized by isolates *S. plymuthica*, *P. chlororaphis* and *P. fluorescens* after seed treatments with 1.2 - 4 × 10⁷ cfu/ml bacterial solutions. Sasse (1997) [20] found that root colonization of rape seed plants in seeds treated with fluorescent pseudomonads (8 × 10⁵ cfu/seed) was 1.8 × 10⁶ and 5.1 × 10⁵ cfu/g root fresh weight after 10 and 30 day respectively. Other authors found that the concentration of *P. fluorescens* was reduced from 10⁶ bis 10⁵ cfu/g root fresh weight after 7 day of plant emergence to 10⁴ bis 10⁵ cfu/g root fresh weight after 60 day of plant growth [28]. The density of rhizospheric bacteria differ strongly according to the root zone of plants as well as the age of the plant [29].

Berg et al. (1996) [9] found that *Bacillus* spp. were able to colonize the roots of *Photoria* after 28 day of growth (10⁴ - 10⁵ cfu/g root fresh weight). In in-vitro experiments Brückner (1998) [28] found that the number of bacterial cells of *B. subtilis* and *P. fluorescens* in the rhizosphere of winter oilseed rape, reduced from 10⁶ and 10⁵ cfu/g root fresh weight after 7 days of planting to 10⁴ and 10³ cfu/g root fresh weight after 60 of planting.

The total number of different bacterial cells was determined in the rhizosphere of OSR plants grown in growth chamber or in greenhouse. An average of 74 × 10⁵ cfu/g root fresh weight was recorded. These results are in agreement with Kleeberger et al. (1983) [30], who recorded viable counts of bacteria of 10⁴ - 10⁵ cfu/g root fresh weight in roots of barley and wheat. Our results are also in agreement with Berg et al. (1996) [9] who determined a bacterial number of 1.5 × 10⁵ cfu/g root fresh weight of OSR plants.

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REFERENCES


Effectiveness of Bacterial and Fungal Isolates to Control *Phoma lingam* on Oilseed Rape *Brassica napus*

_D. R. Fravel, Quarterly Reviews of Phytopathology, Vol. 26, No. 1, 1988, pp. 75-91._


doi:10.1080/07060669209500904


doi:10.1011/j.1365-3059.1985.tb01407.x


doi:10.1094/Phyto-77-190


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