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Effect of seed priming with *Serratia plymuthica* and *Pseudomonas chlororaphis* to control *Leptosphaeria maculans* in different oilseed rape cultivars

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Abstract The efficacy of a seed treatment of oilseed rape (OSR) (*Brassica napus*) with the rhizobacteria *Serratia plymuthica* (strain HRO-C48) and *Pseudomonas chlororaphis* (strain MA 342) applied alone or in combination against the blackleg disease caused by *Leptosphaeria maculans* was tested with different cultivars. Seeds were soaked in bacterial suspensions (bio-priming) to obtain $\log_{10}$ 6–7 CFU seed^{-1}. Cotyledons were inoculated with a 10 ul droplet of *L. maculans* spore suspension of $\log_{10}$ 7 spores ml^{-1} and the disease index (size of lesions) was evaluated 14 days later. A mean disease reduction of 71.6% was recorded for *S. plymuthica* and of 54% for *P. chlororaphis*. The combined treatment was not superior to the treatment with *S. plymuthica* alone. The reduction of the disease caused by *S. plymuthica* was independent of the cultivar's susceptibility, whereas the control effect recorded with *P. chlororaphis* increased with decreasing cultivar resistance to blackleg disease. The bacterial colonization of OSR was restricted to the roots and hypocotyl. No significant difference in bacterial colonization of the rhizosphere was observed between different cultivars, nor between single or combined bacterial seed treatments.

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Introduction

Blackleg disease, caused by *Leptosphaeria maculans* (Desmo.) Ces & de Not. (anamorph *Phoma lingam* Tode ex Fr.) is one of the major diseases of winter and spring cultivars of oilseed rape (*Brassica napus* L. *oleifer*) (Howlett 2004; West et al. 2001). In Europe, annual crop losses reach up to € 118 M in the UK and up to € 154 M in France (Fitt et al. 2006). The infection of oilseed rape (OSR) starts in autumn or spring by air-borne ascospores released from infected stubbles of previous crops. Major symptoms of *L. maculans* infection include cotyledon and leaf lesions during the vegetative phase of the host plant. Stem and crown cankers are the most damaging phase of the disease which cause the major yield losses (Fitt et al. 2006). OSR cultivars are most susceptible to infection in general up to the six-leaf stage. Later infections may lead to lesions further up the stem rather than to crown cankers (Khangura and Barbetti 2001). The severity of stem canker depends on geographic and regional variation of the growing area of OSR, which is directly related to differences in climate, pathogen populations and agronomic practices such as cultivars choice and fungicide use (Aubertot et al. 2006; Stonard et al. 2009). Total destruction of the crop due to seedling death is rare

and usually yield losses at harvest are < 10%, although they can reach 30–50% (West et al. 2001).

The current control strategies depend on the use of resistant cultivars and the application of azole fungicides. Plant resistance is limited (Gaetan 2005; Compant et al. 2005) and fungicides are effective only if correctly timed. Due to fungicide degradation and plant growth producing new, untreated leaves, the control effect is transient and cannot provide long term protection against *L. maculans* infection (West et al. 2002). Often rainfall during autumn prevents entry of heavy application machines in the field and optimal timing of application might be missed.

The objective of this investigation was to test alternative control strategies using seed treatment with naturally occurring, antagonistic microbial biocontrol agents (BCAs). Contrary to the application of fungicides, living microorganisms are able to protect plants because of their capability to colonize the rhizosphere or other parts of the plant (Buchenauer 1998, Berg et al. 2001, Whipps 2001). In addition, their ability to induce systemic resistance in the plant can provide further protection against fungal infection (Compant et al. 2005).

The current study explored the potential of the bacterial BCAs *Pseudomonas chlororaphis* Guignard and Sauvageau (strain MA 342) and *Serratia plymuthica* Lehmann and Neumann (strain HRO-C48) to control leaf infection by *L. maculans* of different OSR cultivars. *P. chlororaphis* (strain MA 342) is the active organism in the registered products Cedomon® and Cerall® (BioAgri AB, Uppsala, Sweden), which have been used on more than 1.5 M hectares against seed- and soil-borne fungal pathogens of barley and wheat, respectively, (Hökeberg 2006). *S. plymuthica* (strain HRO-C48) was developed for control of the soil pathogen *Verticillium dahliae* in strawberries (Berg et al. 2001) and is the active organism in the product RhizoStar® (e-nema GmbH, Raisdorf, Germany). Preliminary investigations in laboratory and greenhouse trials revealed that both bacteria have potential to control *Verticillium longisporum* and *L.maculans* in the OSR cultivar Talent and in a field trail a significant reduction of *L. maculans* leaf infection was recorded after seed bio-priming with *S. plymuthica* (Hammoudi 2007). As *S. plymuthica* cells survive less well on the outer surface of OSR seeds (Müller and Berg 2008), the authors applied the bacteria by bio-priming. Seeds were soaked in a bacterial suspension to allow imbibition of water and bacteria into the seed. Bio-priming seeds provided higher control against *V. longisporum* than coating bacteria on the seed surface (Müller and Berg 2008).

This study included *P. chlororaphis* because the existing registration as a plant protection product would enable a quicker introduction into practice than with *S. plymuthica*, which is not yet registered.

The objective of this investigation was to prove whether the BCAs control *L. maculans* equally well in different OSR cultivars and whether the combined treatment of two BCAs will result in enhanced pathogen control in comparison to single treatments. In addition to the evaluation of the control efficiency of the two BCAs, the potential to colonize plant tissues and the rhizosphere after germination of bio-primed seeds was investigated.

Materials and methods

Plant material

The hybrid lines Tenno, Elektra, Taurus, Talent, Titan and Trabant of *Brassica napus* L. *oleifer* and open pollinated cultivars Aragon, Lorenz, Oase and Billy (Norddeutsche Pflanzenzucht, Hans-Georg Lembke KG, Hohenlieth, Germany) were used. The cultivars are moderately resistant to blackleg disease with a rating of 5 in a scale from 1 (low) to 9 (high resistance) (http://www.rapool.de).

Culture of antagonistic bacteria and seed treatment

Pseudomonas chlororaphis (strain MA 342) was received from Dr. M. Hökeberg, Bioagri, Upsalla, Sweden and *Serratia plymuthica* (strain HRO-C48) from Gabriele Berg, University of Graz, Austria. Oilseed rape seeds were treated with spontaneous rifampicin-resistant mutants of the bacteria produced by Hammoudi (2007). The bacteria were grown in 250 ml Erlenmeyer flasks containing 50 ml TSB (30 g l^{-1} tryptic soy broth) liquid medium (bioMérieux Deutschland GmbH, Nürtingen) at 150 rpm and 28°C for 48 h in the dark. For storage of inoculum, aliquots of 700 µl were transferred into Eppendorf tubes containing 300 µl glycerol and stored at −80°C until use.

Seeds were bio-primed by soaking them in bacterial suspensions. Forty-eight hours after inoculation, the

bacterial suspensions were adjusted to $\log_{10}$ 11 cell ml^{-1} by dilution with 0.85% NaCl. Their density was assessed photometrically (OD at 600 nm) and related to a calibration curve based on colony forming units (CFU) assessed in TSA (TSB supplemented with 1.6% agar). One g of seeds of the different OSR cultivars were treated with 1 ml bacterial suspension and incubated for 5 h at 20°C. When seeds were treated with both antagonists, 0.5 ml of each of the bacterial suspensions was mixed prior to the seed bio-priming. During incubation, seeds were agitated at 150 rpm on a rotary shaker. Seeds were then air dried over night at 20°C.

In order to assure that each seed had between $\log_{10}$ 6–7 CFU $seed^{-1}$, the number of bacteria per seed were assessed by grinding samples of ten seeds for 1 min. in 1 ml 0.85% NaCl using a sterilised mortar and pestle. Suspensions were serially diluted with sterile 0.85% NaCl and plated on to TSA medium supplemented with 100 μg ml^{-1} rifimpicin. Plates were incubated for 48 h at 28°C and the CFU were counted.

Culture of *L. maculans* and plant inoculation

Ascospores of the pathogen *L. maculans* were obtained from residues of several infected OSR plants collected in the field and cultured on potato dextrose agar (Sigma, St. Louis). After purity of cultures had been verified, identification was based on colony pigment and pathogenicity in comparison with *L. biglobosa*. Several fungal isolates were then transferred into one flask containing V-8 medium (200 ml of V-8 juice, 0.75 g of $CaCO_3$ in 800 ml of distilled H_2O) and incubated for 14 days at 24°C on a rotary shaker at 180 rpm (Hammoundi 2007). Conidial suspensions of *L. maculans* for inoculation of OSR were prepared by filtering liquid cultures through four layers of sterile gauze (Paul Hartmann, Wiener Neudorf, Germany). Spores were counted using a haemocytometer and their concentration was adjusted to $\log_{10}$ 7 spores ml^{-1} by dilution with distilled H_2O. Ten days after sowing, the cotyledons were wounded in the centre of each leaf lobe with a sterile needle and 10 μl droplets of the conidial suspension were deposited onto each wound. Control plants were treated in the same way with sterile water. Inoculated plants were covered with plastic sheets to maintain a high humidity for 24 h. For better manifestation of the fungus infection, all leaves except the inoculated cotyledons were removed every 2 days.

Experimental design and disease assessment

Plants were grown in 9-cm diameter plastic pots filled with soil (Einheitserdewerk, Uetersen, Germany). Each pot received three seeds and after germination, plants were removed to obtain a single plant per pot. There were six replicates, each with ten plants on one tray for each control and treatment. The trays were arranged in a randomized block design in a growth chamber with 16/8 h light/dark regime, a temperature of 21/16°C and 80/70% relative humidity (day/night). The experiment was repeated twice. Each experiment contained the following treatments: (1) non-inoculated control (no *L. maculans* and no antagonists); (2) pathogen inoculated control (*L. maculans* only); (3) plants treated with one of the antagonists or the combination of both but no fungal inoculation; (4) *L.maculans*-infested plants treated with one of the antagonist or the combination.

Disease index (DI) according to Borges et al. (2003) was determined 14 days post-inoculation. The disease score was based on the size of 4 lesions per plant (width x length). Symptoms were classified on a scale of 0–6 (0=no symptoms; 1=lesion size <5 mm^2; 2=5–10 mm^2; 3=11–15 mm^2; 4=16–20 mm^2; 5=21–30 mm^2; 6=lesion size >30 mm^2). The disease index was calculated based on 10 plants per tray as $DI=[(n0\times0)+(n1\times1)+\ldots+(n6\times6)\,/\,(N\times6)]\times100$, where n0–n6=the number of plants belonging to classes 0–6, and N=the total number of plants. The mean DI for each treatment was based on the DI of six trays each with ten pots (plants). Experiments were conducted three times. Data on the disease index and amount of healthy plants were *arcsin*-transferred and analysed for variance by ANOVA. For analysis of significant differences between cultivars Tukey's HSD tests at $P=0.01$ was used. Parametric Pearson's rank correlation coefficient with $P=0.05$ was used to compare the efficacy of BCAs and the susceptibility of OSR cultivars. Percentage of healthy plants was also assessed.

Assessment of bacterial colonisation of plants

To test the ability of the bacterial isolates to colonize the rhizosphere and upper parts of OSR plants, whole plants were washed thoroughly under running tap water immediately after disease assessment. Plants were then surface-sterilized for 2 min in 500 ml of 1% sodium hypochlorite (NaOCl) containing 2–3 drops

of 0.02% Tween 20 (Sigma, St. Louis) and rinsed 5 times in sterile distilled water. Leaves, stems, hypocotyl, upper roots and roots from 5 plants were cut and macerated separately using sterilized mortar and pestle. One g samples were transferred into Erlenmeyer flasks containing 10 ml of sterile 0.85% NaCl and serial dilutions were plated on TSA medium supplemented with rifampicin at 100 μg ml^{-1}. Plates were incubated for 48 h at 28°C and CFU g^{-1} plant tissues fresh weight was counted. This investigation was only done with the cultivar Talent and only for the single BCA treatments.

For bacterial enumerations in the rhizosphere, roots with adhering soil were aseptically sampled from five plants and cut into small pieces and transferred to flasks containing 10 ml of sterile 0.85% NaCl. Flasks were shaken at 180 rpm for 2 h before the CFU g^{-1} root fresh weight (RFW) was determined on TSA supplemented with rifampicin. In combined treatments with *S. plymuthica* and *P. chlororaphis*, colonies where distinguished by colony morphology and pigmentation. *S. plymuthica* produces convex colonies with brown-beige pigmentation, whereas *P. chlororaphis* has white and elevated colonies. Data on CFU were $\log_{10}$ transformed and significant differences among treatments were computed by Tukey HSD test at $P=0.01$.

Results

Effect of the BCAs on *L. maculans* infection in different OSR cultivar

In *L. maculans*-infected controls the severity of the infection differed significantly between cultivars ($F=14.4$, df=9, 179, $p<0.0001$). The cultivars Tenno and Elektra were the most resistant with a DI of 37.3 ±14.1 and 38.9±8.4, respectively, whereas Oase was the least resistant with a DI of 70.1±10.5 and only 30% healthy plants (Fig. 1). For cultivars Tenno and Elektra a significantly higher amount of healthy plants was recorded (62.7% ± 14.1 and 61.1% ± 9.6, respectively), compared to the other cultivars tested ($F=16.0$, df=9, 179, $p<0.0001$).

The percent reduction in the DI caused by single or combined seed treatments with *P. chlororaphis* and *S. plymuthica*, together with results on the percentage of healthy plants is presented in Table 1. The average percent DI reduction obtained with *S. plymuthica* (71.6% ± 15.3) was significantly greater than the reduction obtained with *P. chlororaphis* (54.0% ± 20.7), but both single treatments were not significantly different to the combined treatment with a mean effect of 66.1% ± 16.9 ($F=6.9$, df=2, 29, $p<0.004$).

Seeds of cultivars treated with *P. chlororaphis* had reduced DI ($F=8.3$, df=9, 179, $p<0.0001$), with the greatest reduction for the cultivar Oase (71.1% ± 11.2) and the lowest for Tenno, Talent and Elektra (37.7% ± 31.0, 37.0% ± 23.0 and 44.8% ± 32.1, respectively). DI was reduced further for cultivars with seeds treated with *S. plymuthica*, with 85.6% ± 8.1 reduction recorded for cultivar Aragon ($F=4.3$, df=9, 179, $p<0.0001$). Except for cultivars Oase and Billy, the percent disease reduction obtained with *S. plymuthica* always surpassed the reduction caused by *P. chlororaphis*. When seeds were treated with a combination of both BCAs, the average percent of disease reduction was higher than recorded for the same cultivar treated with *P. chlororaphis* alone and lower than in plants treated with *S. plymuthica* alone (except Billy and Oase) ($F=6.6$, df=9, 179, $p<0.0001$). The effect of combined treatment was lowest in cultivars Tenno and Elektra (49.9% ± 28.4 and 56.9% ± 23.2, respectively) and not different between other cultivars. The cultivar Aragon was among the two best performing cultivars in DI reduction as well as in percentage healthy plants in all treatments

When a single BCA was applied, cultivars treated with *S. plymuthica* had a higher amount of healthy plants than those treated with *P. chlororaphis* ($F=12.0$, df=2, 29, $p<0.0001$). Compared to the amount of healthy plants in the untreated controls of 45.8%, all treatments significantly increased the amount of healthy plants ($F=76.9$, df=3, 39, $p<0.0001$).

The correlation between cultivar susceptibility and the disease reduction caused by the BCAs was calculated. A significant positive correlation between cultivar susceptibility to *L.maculans* and the disease reduction obtained by a treatment with *P. chlororaphis* and the combined treatment with both, *P. chlororaphis* and *S. plymuthica* was recorded (p-values=0.019 and 0.008, respectively) (Fig. 2). The linear regression model only explained a relatively small amount of variation between cultivars, in case of *P. chlororaphis* ($r^2=0.52$) and in combined treatment ($r^2=0.60$). A weak positive correlation was also found for treatments with *S. plymuthica* ($r^2=0.26$), however, this correlation was not significant ($p=0.135$).

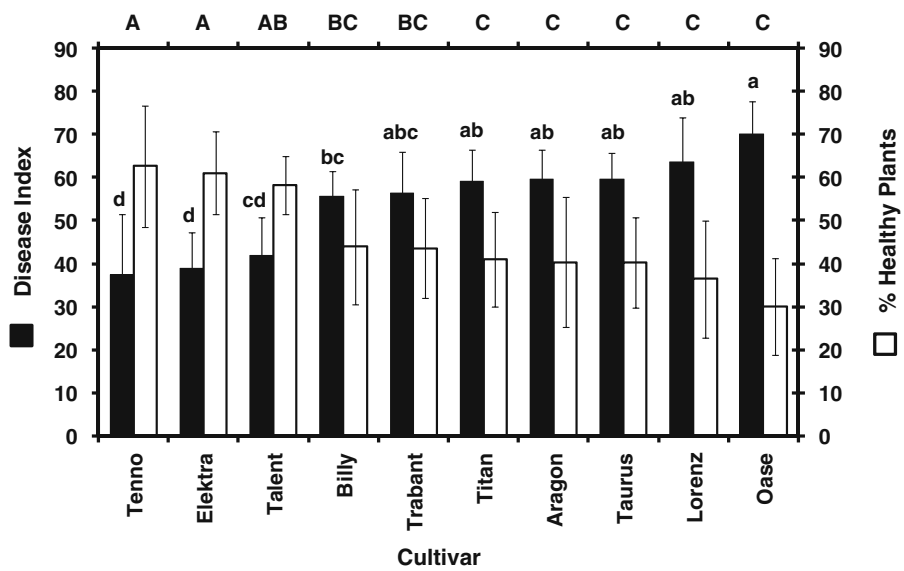

Fig. 1 Disease indices of blackleg disease on selected oilseed rape cultivars inoculated with *Leptosphaeria maculans* suspension (log_{10} 7 spores ml^{-1}, 10 µl cotyledon^{-1}) and the percent of healthy plants in inoculated cultivars. Disease assessment was done 14-days after inoculation. Mean values followed by different letters are significantly different according to Tukeys HSD test at $P \leq 0.01$. Error bars indicate standard deviation

Colonization of plant and rhizosphere by antagonistic bacteria

This study also investigated the ability of the BCAs to colonize the rhizosphere of OSR plants. The difference between cultivars was moderate and not significantly different. No correlation was found between colonization and disease control (data not shown).

Both bacteria applied as seed treatments were absent from stems or leaves of OSR plants (cv. Talent), whether plants had been infected or not infected with *L. maculans*, indicating that bacterial colonization remains confined to the root zone (Fig. 3). In *S. plymuthica*-treated plants, bacterial colonization was restricted to roots, upper roots, and sometimes (i.e., in plants infected with *L.maculans*) to the hypocotyl or lower stem zones. Except for root colonization by *S. plymuthica* (F=5.5, df=1, 53, p>0.023), significantly more *P. chlororaphis* was re-isolated from *L.maculans*-infected than from non-infected plants (F=4.6, df=1, 53, p>0.037).

Table 1 Effect of bacterial strains *Pseudomonas chlororaphis* (PC) and *Serratia plymuthica* (SP) and the combination of both (PC + SP) on the reduction of blackleg disease on different oilseed rape cultivars infected with 10 µl cotyledon^{-1} of a log_{10} 7 spores ml^{-1} suspension of *Leptosphaeria maculans*. Data are presented as percent reduction of untreated controls (DI ± standard deviation) and percent of healthy plants (± standard deviation). Mean values of percent reduction of control in the same column followed by different letters are significantly different according to Tukeys HSD test at $P \leq 0.01$

Cultivar	DI (% reduction of control)			% Healthy plants		
	PC	SP	PC + SP	PC	SP	PC + SP
Tenno	37.7±31.0^{c}	62.5±33.7^{b}	49.9±28.4^{c}	76.8±8.4^{abc}	86.0±9.0^{abc}	80.7±5.4^{ab}
Elektra	44.8±32.1^{cd}	69.8±22.2^{ab}	56.9±23.2^{bc}	78.5±7.3^{ab}	88.2±7.0^{ab}	83.2±5.9^{ab}
Talent	37.0±23.0^{d}	63.4±16.7^{b}	59.7±20.1^{abc}	73.7±6.7^{abc}	84.7±6.4^{abcd}	83.1±7.5^{ab}
Billy	65.0±17.8^{abc}	63.5±17.5^{b}	77.3±12.2^{a}	80.6±6.3^{a}	79.7±6.8^{cd}	87.4±4.2^{a}
Trabant	68.0±11.4^{ab}	76.4±8.1^{ab}	56.9±15.8^{abc}	81.9±6.7^{a}	86.7±4.0^{abcd}	75.6±7.9^{b}
Titan	47.8±25.7^{bcd}	70.9±14.2^{ab}	68.2±12.0^{ab}	71.0±7.9^{bc}	82.7±8.0^{bcd}	81.1±6.4^{ab}
Aragon	68.2±13.4^{ab}	85.6±8.1^{a}	76.2±19.2^{ab}	81.0±6.1^{a}	91.4±2.8^{a}	85.8 ±6.6^{a}
Taurus	51.5±19.5^{abcd}	80.3±9.70^{ab}	72.3±10.8^{ab}	71.0±7.7^{bc}	88.2±5.5^{abc}	83.4±6.8^{ab}
Lorenz	48.9±21.8^{abcd}	74.7±13.0^{ab}	71.2±18.5^{ab}	67.5±9.3^{c}	83.9±5.6^{abcd}	81.5±8.3^{ab}
Oase	71.1±11.2^{a}	68.8±9.8^{ab}	72.3±8.6^{ab}	79.7±7.5^{ab}	78.1±7.6^{d}	80.6±5.8^{ab}
Average ± SD	54.0±20.7	71.6±15.3	66.1±16.9	76.2±7.4	85.0±6.3	82.3±6.5

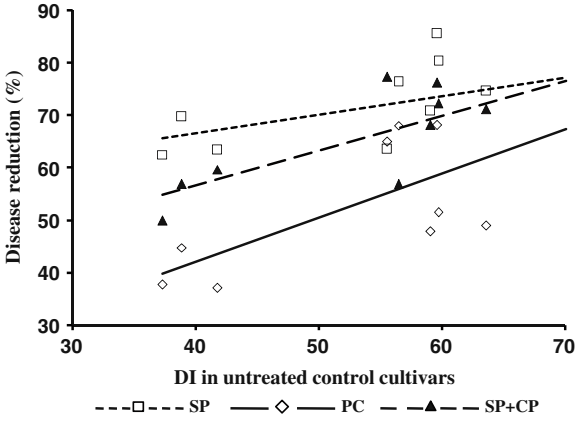

Fig. 2 The relationship between disease severities in different cultivars of oilseed rape inoculated with *Leptosphaeria maculans* suspension (log_{10} 7 spores ml^{-1}. 10 µl $cotyledon^{-1}$) and the percentage of disease control by treatment with *Serratia plymuthica* (SP) (r^2=0.26, p=0.135), *Pseudomonas chlororaphis* (PC) (r^2=0.52, p=0.019) and the combination of both (SP+PC) (r^2=0.60, p=0.008)

The presence of BCAs in the rhizosphere was also analyzed for all cultivars. The initial bacterial cell density was log_{10} 6–7 CFU $seed^{-1}$ for both BCAs. Both, *P. chlororaphis* and *S. plymuthica* were able to efficiently colonize the rhizosphere of *L. maculans*-infected and non-infected OSR seedlings. The average number of bacterial cells was slightly, but not significantly, higher in infected compared to non-infected plants. In the cultivar Titan, treated with *P. chlororaphis*, the bacterial density was significantly higher (F=5.5, df=19, 99, p<0.0001) in *L. maculans*-infected (log_{10} 7.5±0.2 CFU g^{-1} root fresh weight) compared to non-infected plants (6.2±0.4 CFUs g^{-1}). In seeds treated with *S. plymuthica*, rhizosphere colonization was higher, but not significantly different (F=4.3, df=19, 99, p<0.0001) in infected compared to non infected cultivars (Table 2). In plants treated with combinations of *P. chlororaphis* and *S. plymuthica*, there were no significant difference in the bacterial density in the rhizosphere between infected and non-infected plants (Table 3).

Discussion

Although the OSR cultivars used in this study are classified as moderately resistant to the blackleg disease under field conditions, the results indicate variable susceptibility of the cultivars, which might be due to the artificial inoculation with *L. maculans* under controlled conditions or measurement of disease by cotyledon symptoms rather than as stem canker (blackleg) at the stem base.

Several studies have been conducted to evaluate the biocontrol activity of the two BCAs in different plant species. For instance, *P. chlororaphis* MA 342 was found to be a highly effective and constant biocontrol agent against against *Tilletia caries* and *Drechslera* spp. in barley (Hökeberg et al. 1997; Johnsson et al. 1998). Strain HRO-C48 was found to be an effective antagonist of phytopathogenic fungi such as *Verticillium dahliae*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*

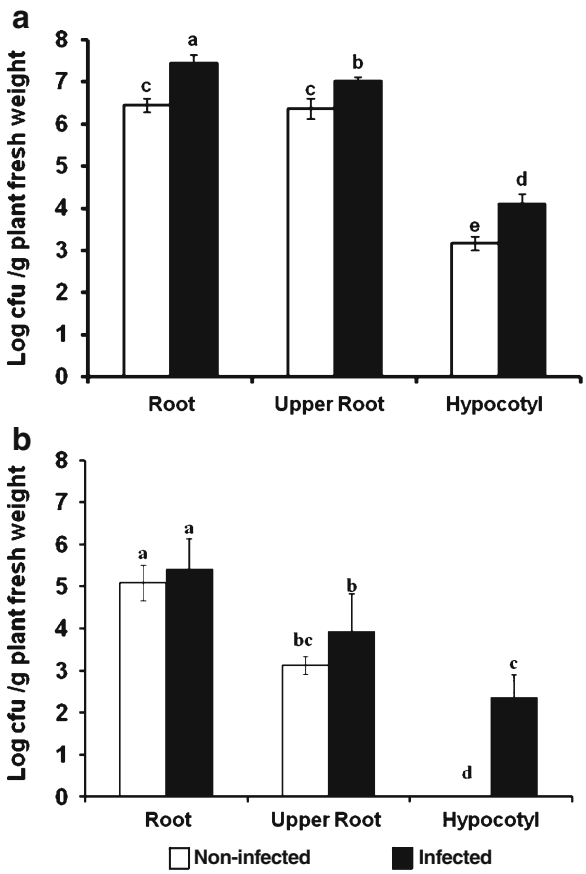

Fig. 3 Abundance of *Pseudomonas chlororaphis* (**a**) and *Serratia plymuthica* (**b**) in different part of oilseed rape plants (cultivar Talent). Seeds were treated with spontaneous rifampicin resistant mutants by bio-priming to obtain log_{10} 6 and 7 CFU $seed^{-1}$. Bacteria were re-isolated after 30 days from infected and non-infected plants with *Leptosphaeria maculans* with 10 µl $cotyledon^{-1}$ of a suspension of log_{10} 7 spores ml^{-1}. Both bacterial isolates were absent from stems or leaves. Mean values followed by different letters are significantly different after Tukeys HSD test at P≤0.01. Error bars indicate standard deviation

Table 2 Population densities of rifampicin resistant *Pseudomonas chlororaphis* (PC) and *Serratia plymuthica* (SP) in the rhizosphere of different oilseed rape cultivars. Bacteria were re-isolated 30 days after sowing from the rhizosphere of infected and non-infected plants with the fungal pathogen *Leptosphaeria maculans* (10 µl cotyledon^{-1} of a $\log_{10}$ 7 spores ml^{-1} suspension). The initial cell density of $\log_{10}$ 6 and 7 CFU seed^{-1} was obtained by bio-priming seeds. Mean values for one bacterium treatment followed by different letters are significantly different according to Tukeys HSD test at $P \leq 0.01$

Cultivar	Log $_{10}$ CFU g^{-1} Root fresh weight			
	PC		SP	
	Non-infected	Infected	Non-infected	Infected
Tenno	6.6±0.2^{abc}	7.1±0.2^{abc}	6.7±0.1^{abc}	7.4±0.1^{abc}
Elektra	6.7±0.3^{abc}	7.6±0.7^{a}	6.4±0.3^{abc}	7.7±0.1^{a}
Talent	6.9±0.2^{abc}	7.4±0.5^{ab}	6.8±0.1^{abc}	7.6±0.1^{ab}
Billy	6.2±0.7^{c}	7.2±0.4^{abc}	6.4±1.2^{abc}	6.6±0.1^{abc}
Trabant	6.2±0.6^{c}	7.2±0.3^{abc}	7.0±0.5^{abc}	7.0±0.1^{abc}
Titan	6.2±0.4^{c}	7.5±0.2^{a}	6.1±0.7^{c}	7.2±0.0^{abc}
Aragon	6.9±0.3^{abc}	7.1±0.4^{abc}	6.1±1.1^{c}	7.1±0.1^{abc}
Taurus	6.9±0.3^{abc}	7.5±0.6^{a}	6.6±0.1^{abc}	7.7±0.1^{a}
Lorenz	6.8±0.2^{abc}	7.1±0.2^{abc}	6.2±1.1^{c}	6.7±0.1^{abc}
Oase	6.3±0.7^{bc}	6.9±0.4^{abc}	6.2±1.2^{bc}	6.9±0.1^{abc}
Average ± SD	6.6±0.4	7.3±0.4	6.5±0.7	7.2±0.1
Increase	0.7±0.0		0.7±0.6	

Table 3 Population densities of rifampicin resistant *Pseudomonas chlororaphis* (PC) and *Serratia plymuthica* (SP) in the rhizosphere of different oilseed rape cultivars after a combined treatment of both bacteria. Bacteria were re-isolated 30 days after sowing from the rhizosphere of infected and non-infected plants with the fungal pathogen *Leptosphaeria maculans* (10 µl cotyledon^{-1} of a $\log_{10}$ 7 spores ml^{-1} suspension). The initial cell density of $\log_{10}$ 6 and 7 CFU seed^{-1} was obtained by bio-priming seeds. Mean values for one bacterium treatment followed by different letters are significantly different according to Tukeys HSD test at $P \leq 0.01$

Cultivar	Log CFU g^{-1} Root fresh weight			
	PC		SP	
	Non-infected	Infected	Non-infected	Infected
Tenno	6.6±0.3^{ab}	7.2±0.1^{ab}	6.6±0.4^{a}	7.1±0.2^{a}
Elektra	6.3±0.5^{ab}	7.0±0.0^{ab}	6.2±0.5^{a}	6.9±0.0^{a}
Talent	6.7±0.3^{ab}	7.3±0.2^{a}	6.7±0.4^{a}	7.1±0.1^{a}
Billy	6.7±0.4^{ab}	7.2±0.1^{ab}	6.7±0.4^{a}	7.1±0.1^{a}
Trabant	6.9±0.4^{ab}	6.7±0.0^{ab}	7.0±0.6^{a}	6.7±0.1^{a}
Titan	6.7±0.3^{ab}	6.9±0.1^{ab}	6.5±0.3^{a}	6.6±0.3^{a}
Aragon	6.1±1.0^{ab}	6.8±0.1^{ab}	6.1±1.0^{a}	6.9±0.1^{a}
Taurus	6.7±0.3^{ab}	7.1±0.1^{ab}	6.6±0.3^{a}	7.0±0.1^{a}
Lorenz	5.9±0.9^{b}	6.8±0.1^{ab}	5.9±1.2^{a}	6.8±0.1^{a}
Oase	6.0±1.6^{ab}	7.1±0.1^{ab}	6.1±1.9^{a}	7.1±0.2^{a}
Average ± SD	6.5±0.6	7.0±0.1	6.4±0.7	6.9±0.1
Increase	0.5±0.6		0.5±0.5	

(Kurze et al. 2001; Frankowski et al. 2001). This study, however, is the first to report a positive control effect on the OSR pathogen *L. maculans*. Seed treatment with *S. plymuthica* and *P. chlororaphis*, whether applied alone or in combination, was able to reduce the disease severity of *L. maculans* in all cultivars. Thus the effect of the micro-organisms is not limited to certain cultivars and they can probably be used successfully also in other OSR cultivars. Significant differences were recorded in the control effect between the cultivars. Usually the relative level of disease control in a single cultivar varied with the BCA applied, except for the cultivar Aragon, which was always among the two cultivars with the greatest response to BCAs. Resistance to plant pathogens is one aim of plant breeding. However, in the future, selection for enhanced interaction with antagonistic rhizobacteria can also be a target for improvement of cultivars.

Although the mode of action of both BCAs is not fully understood, we can suggest that induction of resistance mechanisms in the plant plays a major role in the effect against *L. maculans*, particularly because both BCAs colonize the rhizosphere and are not found in the leaves. We hypothesize that *S. plymuthica* HRO-C48 and *P. chlororaphis* MA 342 were able to suppress the disease due to their ability to induce systemic resistance in the host plant. This was also reported from other strains of *S. plymuthica*. Strain RIGC4 was reported to stimulate defence reactions in cucumber seedlings inoculated with the soil-borne pathogen *Pythium ultimum* (Benhamou et al. 2000). Soil and seed treatment with *S. plymuthica* strain IC1270 induce systemic resistance to *Botrytis cinerea* on tomato and bean leaves and to *Colletotrichum lindemuthianum* on bean (reviewed by De Vleeschauwer and Höfte 2007). Pang et al. (2009) reported that *S. plymuthica* HRO-C48 was able to protect cucumber plants against *Pythium aphanidermatum* damping-off disease and induce the systemic resistance to *Botrytis cinerea* in bean and tomato. *P. chlororaphis* strain O6 induced systemic resistance in tobacco against two foliar bacterial pathogens, the wild fire pathogen *Pseudomonas syringae* pv. *tabaci* and *Erwinia carotovora* subsp. *carotovora* that causes soft rot (Spencer et al. 2003). The spatial separation between the challenging plant pathogen infecting above ground plant parts and the BCAs in our study, strongly suggest that the beneficial protective activity exerted by both bacterial strains is based on activation of the plant's defensive system, rather than being caused by microbial antagonism. Considering that induced resistance is the major effect, then one could expect a relation between general plant resistance against *L. maculans* and the control effect obtained with the BCAs. However, the opposite was found. A positive correlation was recorded between the control obtained with *P. chlororaphis* and the susceptibility of the cultivars. This effect was not recorded with *S. plymuthica*, which provided higher control also in more resistant cultivars. Overall, *S. plymuthica* was superior in the control effect when compared with *P. chlororaphis* and also with the combined treatments. The results clearly indicate that a combined treatment does not have any advantage over the single application of *S. plymuthica*.

P. chlororaphis MA 342 is well known as a strong spermosphere colonizer (Tombolini et al. 1999). In the rhizosphere of onion and carrot seedlings, however, only limited densities (usually below 10 CFU seedling^{-1}) were re-isolated (Bennett and Whipps 2008). Our experiments, however, indicate a higher colonization potential of *P. chlororaphis* MA 342 in the OSR seedling. Interestingly, the effect was consistently recorded in all cultivars, although there was no significant difference in rhizosphere colonization between plants inoculated with *L. maculans* and non-inoculated plants. Rhizosphere colonization was slightly higher in infected compared to non-infected OSR plants, although the difference was not significant.

Although the effectiveness of *S. plymuthica* against *L. maculans* was recorded for the reduction of stem canker in the cultivar Talent and it was successfully tested once in field experiments (Hammoudi 2007), further field trials will be needed to confirm the control effect reported in this investigation. However, the results are promising and can have important implications for integrated biological control of OSR plant diseases for sustainable agriculture. As most cultivars currently in use lack a strong enough resistance against blackleg disease, control measures are necessary. A seed treatment with *S. plymuthica* can minimize the incidence of disease and can provide protection, particularly when fungicide applications cannot be well timed to achieve maximum control. As few bacteria per seed are needed, the bio-priming would be a cost effective control measure to reduce yield losses due to blackleg infection.

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References

Benhamou, N., Gagné, S., Le Quéré, D., & Dehbi, L. (2000). Bacterial-mediated induced resistance in cucumber: beneficial effect of the endophytic bacterium *Serratia plymuthica* on the protection against infection by *Pythium ultimum*. *Phytopathology, 90*, 45–56.

Bennett, A. J., & Whipps, J. M. (2008). Dual application of beneficial microorganisms to seed during drum priming. *Applied Soil Ecology, 38*, 83–89.

Berg, G., Fritze, A., Roskot, N., & Smalla, K. (2001). Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae* Kleb. *Journal of Applied Microbiology, 156*, 75–82.

Borges, A. A., Cools, H. J., & Lucas, J. A. (2003). Menadione sodium bisulphate: a novel plant defence activator which enhances local and systemic resistance to infection by *Leptosphaeria maculans* in oilseed rape. *Plant Pathology, 52*, 429–436.

Buchenauer, H. (1998). Biological control of soil-borne diseases by rhizobacteria. *Journal of Plant Diseases and Protection, 105*, 329–348.

Compant, S., Duffy, B., Nowak, J., Clement, C., & Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principle, mechanisms of action, and future prospects. *Applied and Environmental Microbiology, 71*, 4951–4959.

De Vleeschauwer, D. & Höfte, M. (2007). Using *Serratia plymuthica* to control fungal pathogens of plants. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, 2*, No. 046.

Fitt, B. D. L., Brun, H., Barbetti, M. J., & Rimmer, S. R. (2006). World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *European Journal of Plant Pathology, 114*, 3–15.

Frankowski, J., Lorito, M., Schmid, R., Berg, G., & Bahl, H. (2001). Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. *Archieves of Microbiology, 176*, 421–426.

Gaetan, S. A. (2005). First outbreak of blackleg caused by *Phoma lingam* in commertial canola fields in Argentina. *Plant Disease, 89*, 435.

Hammoudi, O. (2007). Einfluss mikrobieller Antagonisten auf den Befall mit *Phoma lingam* und *Verticillium dahliae* var. *longisporum* an Raps (*Brassica napus* L. var. *napus*). Dissertation, Christian-Albrechts-Universität zu Kiel.

Hökeberg, M. (2006). Development and registration of biocontrol products—experience and perspectives gained from the bacterial seed treatment products Cedomon® and Cerall®. *Proceedings of the International Workshop "Implementation of Biocontrol in Practice in Temperate Regions—Present and Near Future". Research Centre Flakkebjerg, Denmark, November 1–3, 2005. DIAS Report*, 119, p.77.

Hökeberg, M., Gerhardson, B., & Johnsson, L. (1997). Biological control of cereal seed-borne diseases by seed bacterization with greenhouse-selected bacteria. *Eurpean Journal of Plant Pathology, 103*, 25–33.

Howlett, B. J. (2004). Current knowledge of the interaction beween Brassica napus and *Leptosphaeria maculans*. *Canadian Journal of Plant Pathology, 26*, 245–252.

Johnsson, L., Hökeberg, M., & Gerhardson, B. (1998). Performance of the *Pseudomonas chlororaphis* biocontrol agent MA 342 against cereal seed-borne diseases in field experiments. *European Journal of Plant Pathology, 104*, 701–711.

Khangura, R. K., & Barbetti, M. J. (2001). Prevalence of blackleg (*Leptosphaeria maculans*) on canola (*Brassica napus*) in Western Australia. *Australian Journal of Experimental Agriculture, 41*, 1–9.

Kurze, S., Dahl, R., Bahl, H., & Berg, G. (2001). Biological control of soil-borne pathogens in strawberry by *Serratia plymuthica* HRO-C48. *Plant Diseases, 85*, 529–534.

Müller, H., & Berg, G. (2008). Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on Verticillium wilt in oilseed rape. *BioControl, 53*, 905–916.

Pang, Y., Liu, X., Ma, Y., Chernin, L., Berg, G., & Gao, K. (2009). Inducution of systemic resistance, root colonisiation and biocontrol activities of rhizospheric strain of *Serratia plymuthica* are dependent on N-acyl homoserine lactones. *Eurpean Journal of Plant Pathology, 124*, 261–268.

Spencer, M., Ryu, C. M., Yang, K. Y., Kim, Y. C., Kloepper, J. W., & Anderson, A. J. (2003). Induced defence in tobacco by *Pseudomonas chlororaphis* strain O6 involves at least the ethylene pathway. *Physiological and Molecular Plant Pathology, 63*, 27–34.

Tombolini, R., Goag, D. J., Gerhardson, B., & Jansson, J. K. (1999). Colonization pattern of the biocontrol strain *Pseudomonas chlororaphis* strain MA 342 on barely seeds visualized by using green fluorescent protein. *Applied and Environmental Microbiology, 65*, 3674–3680.

West, J. S., Kharbanda, P. D., Barbetti, M. J., & Fitt, B. D. L. (2001). Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology, 50*, 10–27.

West, J. S., Fitt, B. D. L., Leech, P. K., Biddulph, J. E., Huang, Y. J., & Balesdent, M. H. (2002). Effects of timing of *Leptosphaeria maculans* ascospore release and fungicide regime on phoma leaf spot and phoma stem canker development on winter oilseed rape (*Brassica napus*) in southern England. *Plant Pathology, 51*, 454–463.

Whipps, J. (2001). Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany, 52*, 487–511.